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Metastases to bone, lung, and other organs are common and catastrophic consequences of breast cancer progression. There is therefore an urgent need to improve current therapies that address cancer growth and spread. Parathyroid hormone-related protein (PTHrP, also referred to as parathyroid hormone-like protein [PTHLP]) is a secreted factor expressed in almost all normal fetal and adult tissues, acting as an autocrine, paracrine, or intracrine factor in a wide range of developmental and physiological processes. Although PTHrP's dysregulated expression has traditionally been associated with oncogenic pathologies as the major causative agent of malignancy-associated hypercalcemia, recent evidence revealed a driving role in skeletal metastasis progression. Here, we demonstrate that PTHrP is also closely involved in breast cancer initiation, growth and metastasis to bone and lung through mechanisms separate from its bone turnover action, and we suggest that PTHrP as a facilitator of oncogenes could serve as a novel target for therapeutic intervention.

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INTRODUCTION

Parathyroid hormone-related protein (PTHrP) is a secreted factor expressed in almost all normal fetal and adult tissues. It is involved in a wide range of developmental and physiological processes, including serum calcium regulation. PTHrP is also associated with the progression of skeletal metastases, and its dysregulated expression in advanced cancers causes malignancy-associated hypercalcemia. Although PTHrP is frequently expressed by breast tumors and other solid cancers, its involvement in tumor initiation and metastasis *in vivo* is not clear.

BODY

PTHrP is expressed in normal epithelial cells but its expression increases in breast cancer and becomes associated with multiple metastatic lesions and reduced survival. It is, however, still unknown whether PTHrP overexpression is simply a consequence of tumor progression, or whether it is mechanistically linked to the tumor progression process from initiation to metastasis. In order to shed light on this relationship, we ablated the *Pthrp* gene in mammary epithelial cells and determined the consequences of this ablation on tumor initiation, growth and metastasis (1)(Appendix 1).

Toward this objective, we used a model in which *Pthrp* is specifically excised in mammary epithelial cells using the cre-loxP system. In the MMTV-PyMT transgenic mouse model, expression of the oncoprotein polyoma middle T antigen (PyMT) is under the control of the mouse mammary tumor virus long terminal repeat (LTR) and its expression is restricted to the mammary epithelium and absent from myoepithelial and surrounding stromal cells. PyMT, when overexpressed in the mammary epithelium of transgenic mice, it acts as a potent oncogene. Mammary hyperplasia can be detected in this animal model as early as 4 weeks of age. The hyperplasia then progresses to adenoma in 6 weeks, to early carcinoma in 9 weeks and to late carcinoma 12 weeks, with pulmonary metastasis present in 100% of animals. The MMTV-PyMT mouse model of breast cancer is characterized by a high incidence of lung metastasis with highly reproducible progression kinetics. Although PyMT transgenic mice do not develop bone metastasis per se, metastatic cells are found in the bone marrow relatively early and continue to grow in later stages without evidence of bone metastasis.

Disruption of the *Pthrp* gene in the mammary epithelium of the PyVMT transgenic mouse model produces mice that are homozygous (*PyVMT-Pthrpflox/flox*) or heterozygous (*PyVMTPthrpflox/+*) for the floxed *Pthrp* allele. Both groups of animals possess two active *Pthrp* alleles, whether flanked by flox sequences or not. These mice were crossed with a separate strain expressing Cre recombinase under the control of the MMTV promoter that targets Cre expression to the mammary epithelium. Excision of flox-bordered essential *Pthrp* sequences renders the gene nonfunctional. The resulting homozygous mice (*PyVMT-Pthrpflox/flox; Cre+*) therefore express no PTHrP in the mammary epithelium, while the heterozygous mice (*PyVMTPthrpflox/+;Cre+*) present lowered levels of PTHrP expression.

A significant consequence of reduction or elimination of PTHrP expression in the mammary epithelium of the offspring is a marked delay in tumor onset. 100% of control animals (normal PTHrP levels) present tumors by day 55, while heterozygous (PTHrP haploinsufficiency) and homozygous animals (absent PTHrP) reach this percentage by days 77 and 85, respectively. Metastatic spread to lungs was similarly reduced independent of tumor size, illustrating the crucial importance of ablating PTHrP signaling to prevent breast cancer progression and metastasis.

Other Cre/lox studies indicate ablation of floxed sequences in only 90% of the cells, leaving 10% of the cells to potentially express the knock-out protein. In order to overcome the problem of residual PTHrP expression, we isolated cells from *PyVMT Pthrpflox/flox;Cre-* tumors, transfected them with an adenoCre-GFP (or control adeno-GFP) construct, subcultured the cells and purified them by flow cytometry to obtain pure populations of Cre+ (or control Cre-) cells with complete or no *Pthrp* ablation. When these cells were transplanted into the mammary fat pad of healthy syngeneic mice, tumor onset was significantly delayed post-tumor implantation in adeno-Cre animals compared to adeno-GFP controls. Tumor growth was also significantly delayed in the adenoCre mice. Metastatic tumor cells were detectable in the bone marrow of adenoCre animals during killing, confirming that this model can be used to examine natural progression of breast cancer from the primary site to the skeleton.

The ablation of *Pthrp* was also observed to inhibit G0/G1 to S transition in tumor cells, to enhance tumor cell apoptosis (increased TUNEL staining and decreased Bcl-2 expression) and to decrease Akt1 and increase Akt2 expression. The reduction in metastases may have been related to reductions in the expression

of CXC chemokine receptor 4 and of angiogenesis. Finally, a blocking antibody to PTHrP, inhibited primary tumor growth and lung metastases in a xenograft model.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated in mice the pleiotropic involvement of PTHrP in key steps of breast cancer initiation, progression, and metastasis
- The MMTV-Cre transgene was used to target the *Pthrp* gene in mouse mammary epithelial cells
- Loss of PTHrP expression did not affect tumor incidence, but it did dramatically prolong tumor latency, slow tumor growth, and reduce metastases in lung and bone
- Restraint of tumor growth correlated with reduced proliferation and increased apoptosis, due to alterations in cyclin D1, protein kinase B 1 and 2, and B-cell lymphoma 2 expression
- The reduction in metastases was related to reductions in the expression of CXC chemokine receptor 4 and the inhibition of angiogenesis
- A blocking antibody to PTHrP inhibited primary tumor growth and metastases in a xenograft model.

REPORTABLE OUTCOMES

- Li, J., Karaplis, A.C., Huang, D.C., Siegel, P.M., Camirand, A., Yang, X.F., Muller, W.J., and Kremer, R. 2011. PTHrP drives breast tumor initiation, progression, and metastasis in mice and is a potential therapy target. *J Clin Invest* 121:4655-4669.

- Kremer, R., Li, J., Camirand, A., and Karaplis, A.C. 2011. Parathyroid hormone related protein (PTHrP) in tumor progression. *Adv Exp Med Biol* 720:145-160.

CONCLUSIONS

We have shown that PTHrP is involved in breast cancer initiation, growth and metastasis. Our findings suggest that PTHrP plays a role in a number of critical checkpoints for PyVMT, which points to a novel role as a facilitator of oncogenes and emphasizes the importance of attempting its targeting for therapeutic purposes (2)(Appendix 2).

The clinical relevance of this work in mice was more recently substantiated in humans. In work published in *Nature Genetics*, Ghoussaini and colleagues combined several datasets encompassing 70,000 patients and 68,000 controls in order to perform genome-wide association studies to identify new breast cancer susceptibility loci (3). One of the three new loci they identified, rs10771399, was in a 300 kb linkage disequilibrium block that contains only one gene, *PTHLH (PTHRP)*. If the *PTHLH* gene is confirmed to be causal in resequencing studies of the rs10771399 locus, the animal studies described in the present work will be instrumental in understanding how PTHrP might alter disease susceptibility (4).

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APPENDICES

Appendix 1 (manuscript)
Li, J., Karaplis, A.C., Huang, D.C., Siegel, P.M., Camirand, A., Yang, X.F., Muller, W.J., and Kremer, R. 2011. PTHrP drives breast tumor initiation, progression, and metastasis in mice and is a potential therapy target. *J Clin Invest* 121:4655-4669.

Appendix 2 (manuscript)
Kremer, R., Li, J., Camirand, A., and Karaplis, A.C. 2011. Parathyroid hormone related protein (PTHrP) in tumor progression. *Adv Exp Med Biol* 720:145-160.

PTHrP drives breast tumor initiation, progression, and metastasis in mice and is a potential therapy target

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Parathyroid hormone–related protein (PTHrP) is a secreted factor expressed in almost all normal fetal and adult tissues. It is involved in a wide range of developmental and physiological processes, including serum calcium regulation. PTHrP is also associated with the progression of skeletal metastases, and its dysregulated expression in advanced cancers causes malignancy-associated hypercalcemia. Although PTHrP is frequently expressed by breast tumors and other solid cancers, its effects on tumor progression are unclear. Here, we demonstrate in mice pleiotropic involvement of PTHrP in key steps of breast cancer — it influences the initiation and progression of primary tumors and metastases. *Pthrp* ablation in the mammary epithelium of the PyMT-MMTV breast cancer mouse model caused a delay in primary tumor initiation, inhibited tumor progression, and reduced metastasis to distal sites. Mechanistically, it reduced expression of molecular markers of cell proliferation (Ki67) and angiogenesis (factor VIII), antiapoptotic factor Bcl-2, cell-cycle progression regulator cyclin D1, and survival factor AKT1. PTHrP also influenced expression of the adhesion factor CXCR4, and coexpression of PTHrP and CXCR4 was crucial for metastatic spread. Importantly, PTHrP-specific neutralizing antibodies slowed the progression and metastasis of human breast cancer xenografts. Our data identify what we believe to be new functions for PTHrP in several key steps of breast cancer and suggest that PTHrP may constitute a novel target for therapeutic intervention.

Introduction

Metastases to bone, lung, and other organs are common and catastrophic consequences of breast cancer progression; most patients do not die from the primary tumor, but because of cancerous invasion to distal sites (1, 2). Once breast cancer metastases are established in bone or lung, the condition is generally considered incurable. There is therefore an urgent need to improve current therapies that address cancer spread, and an ideal solution will target upstream signaling molecules to prevent compensatory mechanisms that can result from blockade of individual downstream signaling points (3, 4).

Parathyroid hormone–related protein (PTHrP, also referred to as parathyroid hormone–like protein [PTHLP]) is a secreted factor expressed in almost all normal fetal and adult tissues. The 13 N-terminal amino acids of PTHrP are highly homologous to those of parathyroid hormone (PTH), a characteristic that allows PTHrP to act through the type 1 PTH receptor (PTH1R) (5). The rest of the PTHrP amino acid sequence is unique, however, and confers to the molecule many properties resulting from signal transduction cascades and nuclear translocation distinct from those of PTH (6). PTHrP acts as an autocrine, paracrine, or intracrine factor in a wide range of developmental and physiological processes (7, 8), it has growth-promoting and antiapoptotic properties (6), and it plays a crucial role in the development of the mammary gland and skeleton (8–10). Of special interest is the association of PTHrP with oncologic pathologies such as breast cancer (11, 12) and lung (13–15), prostate (16–18), renal (19), colorectal (20–22), skin (23, 24), and gastric carcinomas (25, 26). Circulating levels of PTHrP generally correlate with the more advanced stages of cancer (20, 27–32), and PTHrP regulates the expression of several tumor-relevant genes (33). Despite the frequent association of PTHrP dysregulation with many tumor types, a precise and direct role for PTHrP in cancer development and progression has been difficult to prove, and its involvement in tumor initiation in vivo and in critical steps of malignant conversion is not clear.

Here, we demonstrate PTHrP implication in key steps of breast cancer initiation, progression, and metastasis. We show that PTHrP plays a major role in stimulation of breast tumor growth rates and metastatic spread to distal organs through its effects on several crucial control molecules, including prosurvival signal molecule AKT and chemokine receptor CXCR4.

Results

Pthrp ablation occurring after birth allows normal mammary development. To clarify the role of PTHrP in tumorigenesis, the human breast cancer mouse model PyMT-MMTV (where the mT oncogene drives oncogenic transformation; ref. 34) was used to generate animals with a Cre-loxP–mediated (35) hetero- or homozygous *Pthrp* gene ablation specifically targeted to the mammary epithelium (ME) (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI46134DS1). All animals used in the present study were confirmed by marker analysis to possess more than 99% FVB/NJ background. In standard PyMT-MMTV animals, tumors appeared spontaneously, approximately 100% of these tumors expressed PTHrP (55 tumors tested by RT-PCR), and their PTHrP expression increased with age (Figure 1A). In contrast, in *Pthrp*-ablated animals, spontaneous breast tumors showed

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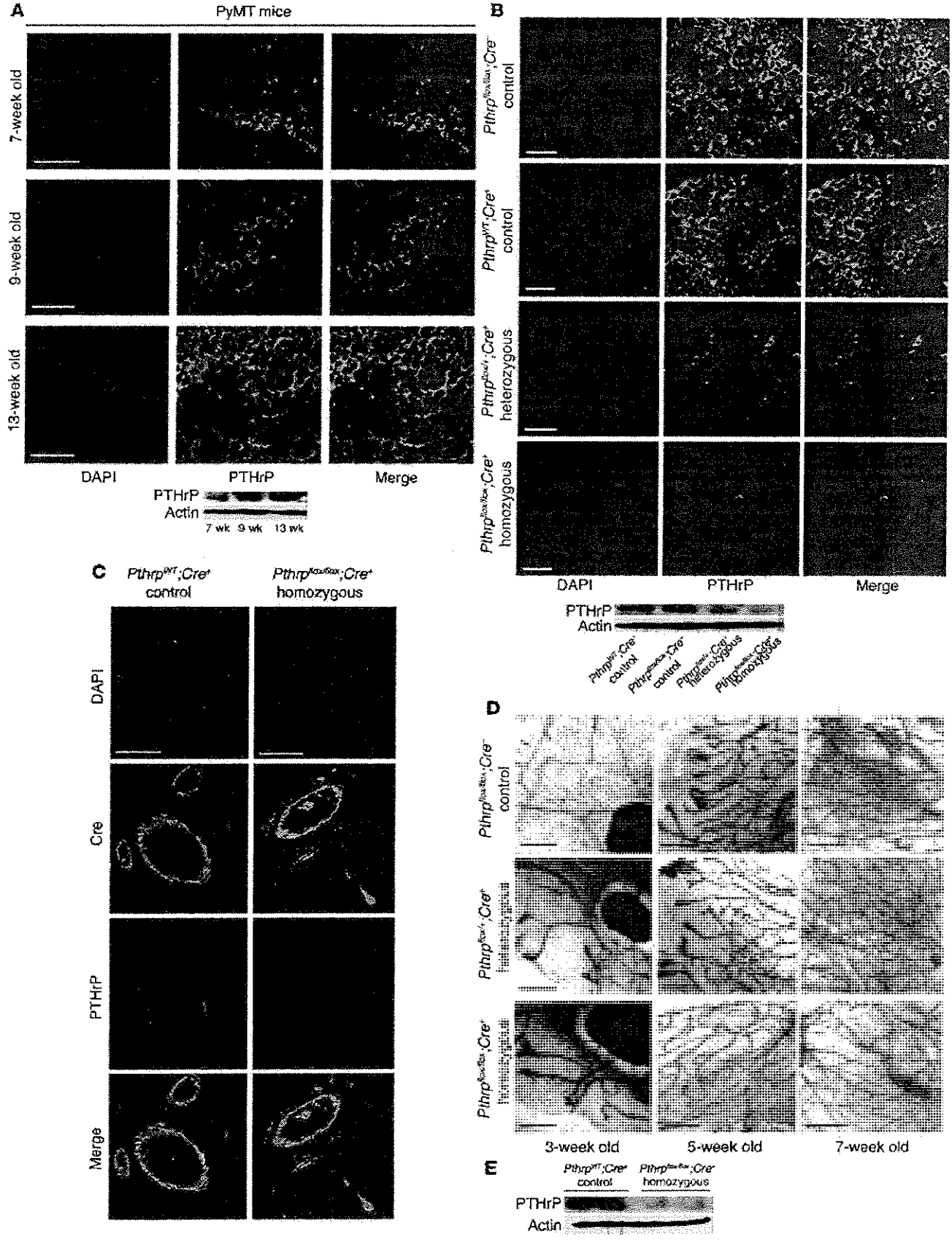
A
PyMT mice
7-week old
9-week old
13-week old
DAPI
PTHrP
Merge
PTHrP
Actin
7 wk 9 wk 13 wk
B
Pthrp flox/flox;Cre- control
Pthrp WT;Cre+ control
Pthrp flox/+;Cre+ heterozygous
Pthrp flox/flox;Cre+ homozygous
DAPI
PTHrP
Merge
PTHrP
Actin
Pthrp WT;Cre+ control
Pthrp flox/flox;Cre+ control
Pthrp flox/+;Cre+ heterozygous
Pthrp flox/flox;Cre+ homozygous
C
Pthrp WT;Cre+ control
Pthrp flox/flox;Cre+ homozygous
DAPI
Cre
PTHrP
Merge
D
Pthrp flox/flox;Cre- control
Pthrp flox/+;Cre+ heterozygous
Pthrp flox/flox;Cre+ homozygous
3-week old
5-week old
7-week old
E
Pthrp WT;Cre+ control
Pthrp flox/flox;Cre+ homozygous
PTHrP
Actin

Figure 1
Cre-mediated Pthrp ablation in ME allows normal mammary development. (**A**) Confocal images of IF staining with anti-PTHrP antibody in spontaneous breast tumors from standard PyMT mice and Western blot quantification showing increasing PTHrP expression in these tumors with respect to age. (**B**) Confocal images of IF staining for DAPI (blue) and PTHrP (green) in tumor tissues from control (*Pthrp*$^{flox/flox}$;*Cre*$^{-}$ and *Pthrp*^{WT};*Cre*$^{+}$), heterozygous (*Pthrp*$^{flox/+}$;*Cre*$^{+}$), and homozygous (*Pthrp*$^{flox/flox}$;*Cre*$^{+}$) transgenic animals showing incremental decrease in PTHrP expression with allele ablation. Lower panel: Western blot quantification for PTHrP expression in tumors from the various genotypes. (**C**) Confocal images of IF staining with DAPI (blue), Cre recombinase (green), and PTHrP (red) showing colocalization of Cre and PTHrP. (**D**) Whole-mount staining analysis (Neutral Red) of mammary glands showing ductal outgrowth at 3, 5, and 7 weeks for control, heterozygous, and homozygous female virgin mice. (**E**) Western blot of PTHrP expression in mammary glands of virgin 7-week-old mice. Scale bars: 50 μm (**A–C**); 5 μm (**D**).

incremental reduction in PTHrP expression from *Pthrp*$^{flox/flox}$;*Cre*$^{-}$ (control) to *Pthrp*$^{flox/+}$;*Cre*$^{+}$ (heterozygous) to *Pthrp*$^{flox/flox}$;*Cre*$^{+}$ (homozygous) (Figure 1B). *Pthrp*$^{WT/WT}$;*Cre*$^{+}$ animals were generated to test potential artifactual side effects caused by expression of Cre recombinase, but showed no difference from other controls throughout all experiments.

When tumor cells were cultured in vitro, radioimmunometric assays for PTHrP in the conditioned medium indicated low residual expression of PTHrP in homozygous ablated cells as follows: *Cre*$^{-}$ control cells, 178.7 ± 33.6 pg/ml; *Cre*$^{+}$ tumor-derived cells, 10.1 ± 2.3 pg/ml; mean ± SD, n = 13 and 10 mice, respectively. In tumor-bearing mice, circulating PTHrP was undetectable, and calcium serum concentrations were not significantly different between control (2.28 ± 0.39 mmol/l) and *Pthrp*-ablated mice (2.23 ± 0.23 mmol/l).

PTHrP expression in the normal gland was localized both in luminal epithelial cells and in myoepithelial cells (Supplemental Figure 2, A–C, and ref. 36). PTH1R expression was unaffected by *Pthrp* ablation (Supplemental Figure 3).

Immunofluorescence (IF) staining confirmed Cre expression in the luminal epithelium of control mice (Figure 1C). The *Cre* gene was expressed under the control of the MMTV-long terminal repeat (MMTV-LTR), and its expression was detectable from 6 days postpartum (37). *Pthrp* ablation therefore occurred after birth, and mammary glands from 3-, 5-, and 7-week-old virgin females presented normal ductal outgrowth as well as normal lactation capacity with no detectable differences among genotypes (Figure 1D). PTHrP in the mammary gland was substantially reduced in 7-week-old homozygous mice in comparison with control animals (Figure 1E). These data show that ME-targeted *Pthrp* deletion can be achieved without hindering normal mammary gland development.

Pthrp ablation delays breast cancer initiation and primary tumor progression. Tumor hyperplasia was detectable in the mammary gland of control animals as early as 27 days afrer birth, adenomas were visible by 35 days, and early adenocarcinomas were visible by day 45 (Figure 2A). In contrast, ablation of both *Pthrp* alleles significantly delayed tumor initiation (Figure 2A). Tumor growth over time was reduced in homozygous *Pthrp*$^{flox/flox}$;*Cre*$^{+}$ animals in comparison with *Pthrp*$^{flox/flox}$;*Cre*$^{-}$ and *Pthrp*^{WT};*Cre*$^{+}$ controls (Figure 2B), and tumor weight/mouse at sacrifice (13 weeks) was 70% lower in ablated animals than in controls (Figure 2C). While 100% of control mice presented palpable tumors (2-mm diameter) around day 55, heterozygous and homozygous animals reached this percentage by days 75 and 92, respectively (Figure 2D). Homozygous mice reached tumor size requiring humane sacrifice at a much later age than control and heterozygous mice (Figure 2E). Tumors from hetero- and homozygous mice were 50% and 75% smaller than their respective *Cre*$^{-}$ controls, and there were 35% and 60% fewer tumors in heterozygous and homozygous mice at 13 weeks (Figure 3, A and B, and Supplemental Table 1). The expression of Cre was not involved in the changes observed in tumor delay, as demonstrated by the *Pthrp*^{WT};*Cre*$^{+}$ controls, which did not differ from the other controls (Figure 2, B–E, and Figure 3, A and B). These results show that ablation of the *Pthrp* gene in the ME of PyMT-MMTV mice significantly delays primary breast cancer initiation events, even before tumors reach a palpable stage, and reduces their subsequent growth.

More complete Pthrp ablation by Cre-carrying adenovirus further delays breast cancer initiation and progression. Because some residual PTHrP expression was detectable in approximately 20% of primary tumors from *Pthrp*$^{flox/flox}$;*Cre*$^{+}$ animals (Figure 1B), a more complete *Pthrp* knockout was achieved by transfecting isolated cells from *Pthrp*$^{flox/flox}$;*Cre*$^{-}$, *Pthrp*$^{flox/+}$;*Cre*$^{-}$ and *Pthrp*^{WT} tumors with an adenovirus containing a CreGFP sequence or with a control adenovirus containing GFP only. Transfected adenoGFP control or adenoCre^{+} cells purified by cell sorting were transplanted into the fourth mammary fat pad (MFP) of healthy FVB syngeneic mice (5 × 10^5 cells). PTHrP expression was eliminated in the adenoCre^{+}-derived tumors (Figure 4, A and B), tumor volume was greatly decreased (Figure 4C), and tumor load at injection site was dramatically reduced (Figure 4, D and E). These results show that more stringent *Pthrp* ablation conditions enhance tumor inhibition.

Pthrp ablation modifies cell-cycle, apoptosis, and angiogenesis events. Decreasing PTHrP exptession reduced Ki67 (cell proliferation), factor VIII (angiogenesis), and cyclin D1 staining (Figure 5, A and B). Propidium iodide flow cytometry analysis revealed that 53.7% ± 1.19% of control *Pthrp*$^{flox/flox}$;*Cre*$^{-}$ remained in G_0/G_1 compared with 71.32% ± 3.70% of homozygous *Pthrp*$^{flox/flox}$;*Cre*$^{+}$. In contrast, 18.34% ± 1.80% of conrol *Pthrp*$^{flox/flox}$;*Cre*$^{-}$ and 8.10% ± 4.00% of homozygous *Pthrp*$^{flox/flox}$;*Cre*$^{+}$ tumor cells were in S phase, an observation consistent with the cyclin D1 decrease (not shown). Importantly, in cultured cells isolated from tumors, cyclin D1 expression colocalized specifically with cells that escaped PTHrP ablation (Figure 5C), suggesting that in this system, PTHrP is crucial for cyclin D1 expression. *Pthrp* ablation was accompanied by an increase in TUNEL apoptotic staining in tumors and tumor-derived cultured cells (Figure 5D) and by a decrease in Bcl-2 expression (Figure 5B). Western blots showed no difference in expression levels for factor VIII, cyclin D1, and Bcl-2 between *Pthrp*$^{flox/flox}$;*Cre*$^{-}$ and *Pthrp*^{WT};*Cre*$^{+}$ controls (Supplemental Figure 4), indicating that decreases in signaling molecule levels can be attributed to *Pthrp* ablation. Overall, these data indicate a pleiotropic effect of PTHrP in cell-cycle, apoprosis, and angiogenesis events.

PTHrP is involved in CXCR4 and AKT expression control. Pthrp ablation was accompanied by inhibition of metastasis marker CXCR4 expression in primary breast tumors of the same size (controls: 13 weeks; homozygous animals: 18 weeks; Figure 6, A and B). Furthermore, CXCR4 in cells isolated from primary tumors appeared exclusively in cells that avoided *Pthrp* ablation (Figure 6B). *Pthrp* ablation also decreased AKT1 total protein and increased total AKT2 in tumor tissues (Figure 6, C and D), and

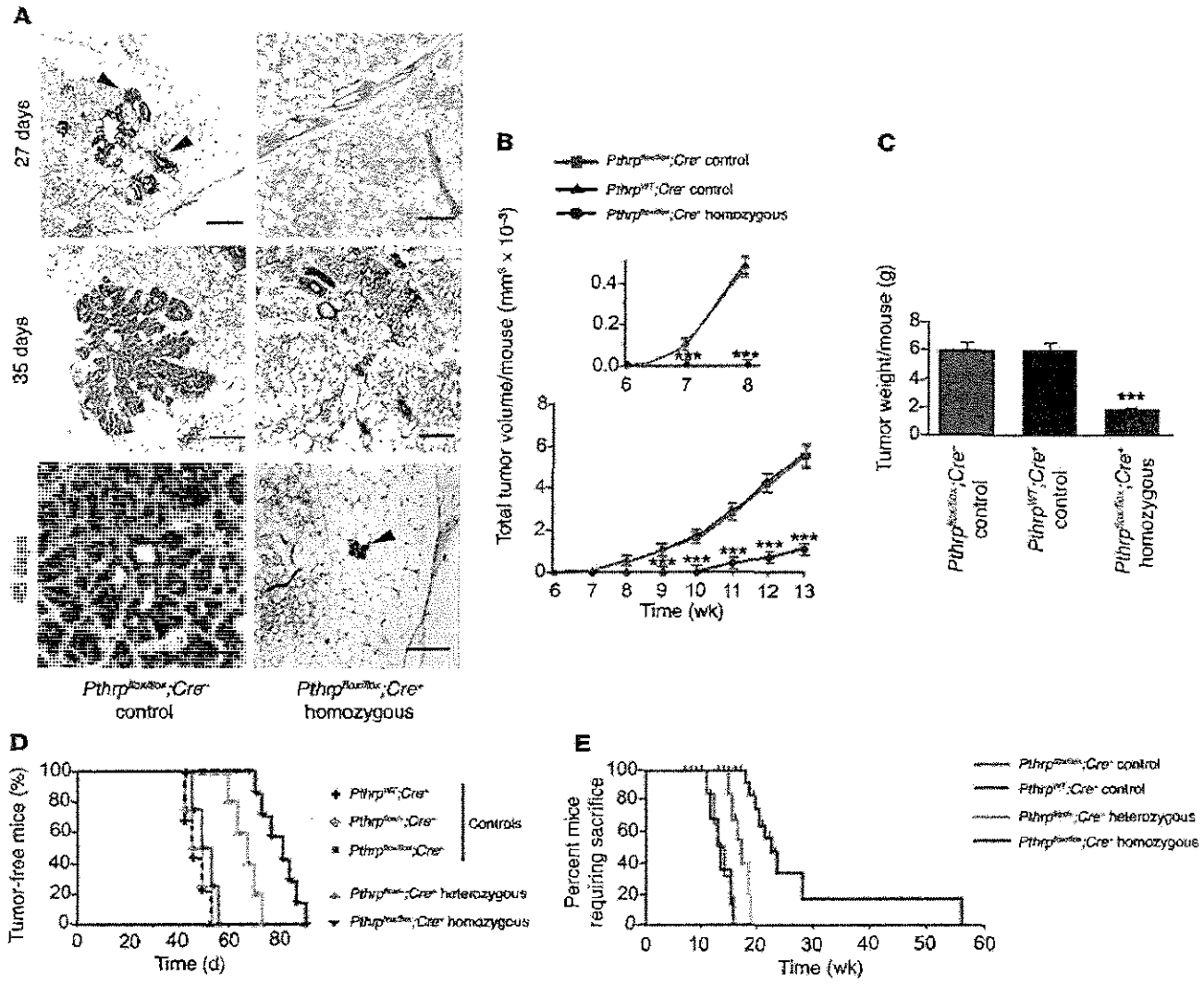

Figure 2

Pthrp ablation delays breast cancer initiation and progression. (**A**) H&E staining of breast tissues from control (*Pthrp*$^{flox/flox}$;*Cre*$^{-}$) and homozygous (*Pthrp*$^{flox/flox}$;*Cre*$^{+}$) animals at 27, 35, and 45 days. Hyperplasias (arrowheads) are not detectable in homozygous animals before 45 days. Scale bars: 200 μm. (**B**) Tumor volume over time for control (*Pthrp*$^{flox/flox}$;*Cre*$^{-}$ and *Pthrp*^{WT};*Cre*$^{+}$) and homozygous mice (*Pthrp*$^{flox/flox}$;*Cre*$^{+}$) showing that the delay in tumor growth is not related to the expression of the *Cre* gene. Inset: early time points. Values shown represent mean ± SD, $n = 12$ mice per group. ***$P < 0.001$. (**C**) Tumor weight per mouse at sacrifice (13 weeks) for control and homozygous animals. Values shown represent mean ± SD, $n = 12$ mice per group. ***$P < 0.001$. (**D**) Kaplan-Meier analysis of tumor onset for mice of all genotypes illustrating allelic effect for *Pthrp* ablation. (**E**) Kaplan-Meier analysis showing that control mice reach age requiring sacrifice much earlier than homozygous animals.

most residual AKT1 colocated with residual PTHrP in *Cre*$^{+}$ cultured cells isolated from tumors (Figure 6E). Again, the expression of Cre was not involved in changes seen for AKT or CXCR4 (Supplemental Figure 4). Phosphorylation of AKT1 (Ser473) was also inhibited by *Pthrp* ablation (Figure 6F). siRNA experiments targeting AKT1 effectively decreased its levels in control (Cre^{-}) and *Pthrp*-ablated (Cre^{+}) tumor cells (Figure 7A). Cell proliferation was reduced by 38.9% ± 8.0% by *Pthrp* ablation alone (Figure 7B), by 46.7% ± 4.1% through AKT1 knockdown alone, and by 80.2% ± 2.2% in *Pthrp*-ablated cells combined with AKT1 siRNA. These data indicate that PTHrP is involved in the control of expression of survival molecules AKT1 and AKT2 and of the chemokine receptor CXCR4.

PTHrP drives metastatic spread to peripheral blood, bone marrow, and lungs, and distal metastases comprise PTHrP/CXCR4 double-positive cells. Cells from control tumors possessed more than twice the Matrigel invasiveness potential of cells from *Pthrp*-ablated mice (Figure 8A), and their motility after surface wounding was higher (Figure 8B). There were fewer circulating tumor cells in homozygous than in control animals (same-size tumors, controls: 13 weeks; homozygous: 18 weeks; Figure 8C). Tumor cells flushed from bone marrow were detectable only in control animals (average of 31.5 ± 3.4 cells per animal, $n = 4$; Figure 8D).

Similarly, the incremental decrease in PTHrP lowered the number of micro- and macroscopic metastatic lesions in lung tissues. At 13 weeks, 100% (45/45) of control mice presented lung metastases compared

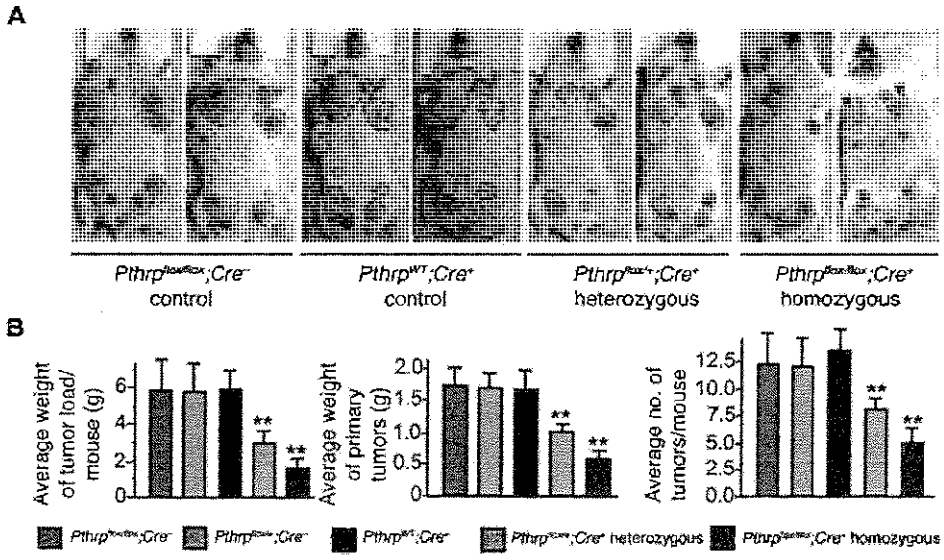

Figure 3
Pthrp ablation reduces spontaneous tumor load. (**A**) Tumor load in whole animals (13 weeks). (**B**) Average weight of tumor load per mouse, average weight of primary tumors, and average number of tumors per mouse at sacrifice for the same genotypes. Values shown represent mean ± SD; $n = 20$ for *Pthrp*$^{flox/flox}$;*Cre*$^-$, $n = 18$ for *Pthrp*$^{flox/+}$;*Cre*$^-$, $n = 22$ for *Pthrp*$^{WT/WT}$;*Cre*$^+$ (controls), $n = 12$ for *Pthrp*$^{flox/+}$;*Cre*$^+$ (heterozygous), and $n = 30$ for *Pthrp*$^{flox/flox}$;*Cre*$^+$ (homozygous), $^{**}P < 0.01$.

with 47% (8/17) for heterozygous and 0% for homozygous animals (0/18) (Figure 8E). At 18 weeks, when same-size tumors were achieved in homozygous mice, smaller lung tumors appeared in 40% (12/30) of homozygous mice compared with 100% of control mice euthanized at 13 weeks for humane reasons (Figure 8, F and G). When tumor cells were transfected with adenoCreGFP or adenoGFP virus, purified, and implanted (5×10^5 cells) in the fourth MFP of syngeneic mice, same-size primary tumors in the MFP were achieved at 8 weeks in control, 11 weeks in heterozygous, and 16 weeks in homozygous mice (not shown). At those stages, lung metastases were present in 70% (16/23) of control, 55% (6/11) of heterozygous, and 0% (0/22) of homozygous mice (Figure 8, H and I).

Importantly, in the spontaneous PyMT tumor model, lung metastases cells in control and homozygous mice (same-size rumors, 13 weeks and 18 weeks, respectively) were almost uniformly PTHrP and CXCR4 positive (Figure 9), indicating that lung metastases were mainly derived from PTHrP-expressing tumor cells that escaped gene ablation (Supplemental Figure 5). These results point to a driving role for PTHrP in invasion and metastasis in this system and suggest that combined expression of PTHrP and CXCR4 is crucial to metastatic spread.

Anti-PTHrP neutralizing mAbs inhibit breast cancer progression in vitro and in vivo. mAbs were produced against human $PTHrP_{1-33}$, and 2 molecules positive exclusively for this peptide (158 IgG, M45 IgM) significantly inhibited Matrigel invasion by human MDA-MB-435 breast cancer cells (PTHrP positive, secreting 300±30 pg/ml of PTHrP in the conditioned medium) compared with cells treated with a control antibody (Figure 10A). MDA-MB-435 cells were also injected into the MFPs of BALB/c nu/nu mice and animals treated with the mAbs (200 μg subcutaneously every 48 hours for 6 weeks). Primary tumor growth in untreated animals reached 1.5–2.0 cm^3 by 6 weeks after tumor transplantation. A significant inhibition ($P < 0.01$) in the size of primary tumor growth was observed in mice treated with 158 or M45 compared with controls (Figure 10B). The 158 antibody reduced the expression of CXCR4 and AKT1 in mammary tumors developing from injected cells (Figure 10C). After 6 weeks, approximately 100% of control animals presented lung metastases in contrast with 33% for mAb-treated mice (Figure 10, D and E), and metastases from lungs of antibody-treated mice were fewer and 50% smaller than those from vehicle-treated controls (Figure 10, F and G). CXCR4 expression was still prevalent in lung metastases despite antibody treatment (Figure 10H), suggesting that either efficacy of PTHrP neutralization was not optimized or that CXCR4 expression can be driven by additional factors. Liver and kidney tissues from animals treated with antibodies presented no hisrological evidence for toxicity due to treatment (not shown). These results suggest that anti-PTHrP antibodies can be considered for therapeutic use against human breast cancer progression.

Overall, our results indicate that PTHrP favors tumor cell survival in the primary site and plays a role in breast cancer progression through its control of cyclin D1, Ki67, Bcl-2, factor VIII, AKT1, AKT2, and CXCR4 levels (Figure 11). When tumor cells migrate to distal sites, adhesion to local ligands is facilitated by PTHrP's maintenance of CXCR4 levels, and its positive action on tumor proliferation allows consequent metastatic expansion of PTHrP/CXCR4 double-positive cells.

Discussion

To investigate the involvement of PTHrP in breast cancer initiation, progression, and metastasis, we constructed an ME-specific *Pthrp*-knockout animal in the PyMT-MMTV transgenic mouse model of breast tumorigenesis, using mice homozygous for a floxed *Pthrp* allele (2 *loxP* sites flanking exon 4, which encodes most of the PTHrP protein) (38). The PyMT mouse is a model with complete penetrance and approximates the 4 identifiable stages of tumor progression observed in human breast tumors (hyperplasia, adenoma, early carcinoma, and late carcinoma). These stages are followed in PyMT animals by a high frequency of distal metastasis, and the morphological characteristics and expression of biomarkers parallel those in the human cancer process (39). We obtained mice presenting various degrees of ablation of the *Pthrp* gene, demonstrating that reduction of PTHrP expression in ME brings substantial delays in breast cancer initiation without affecting mammary development. In *Pthrp*-ablated animals, primary tumor hyperplasia was considerably delayed, and palpable tumors appeared much later and were smaller and fewer than in control mice. Our observations are in agreement with several clinical studies in which patients with tumors produc-

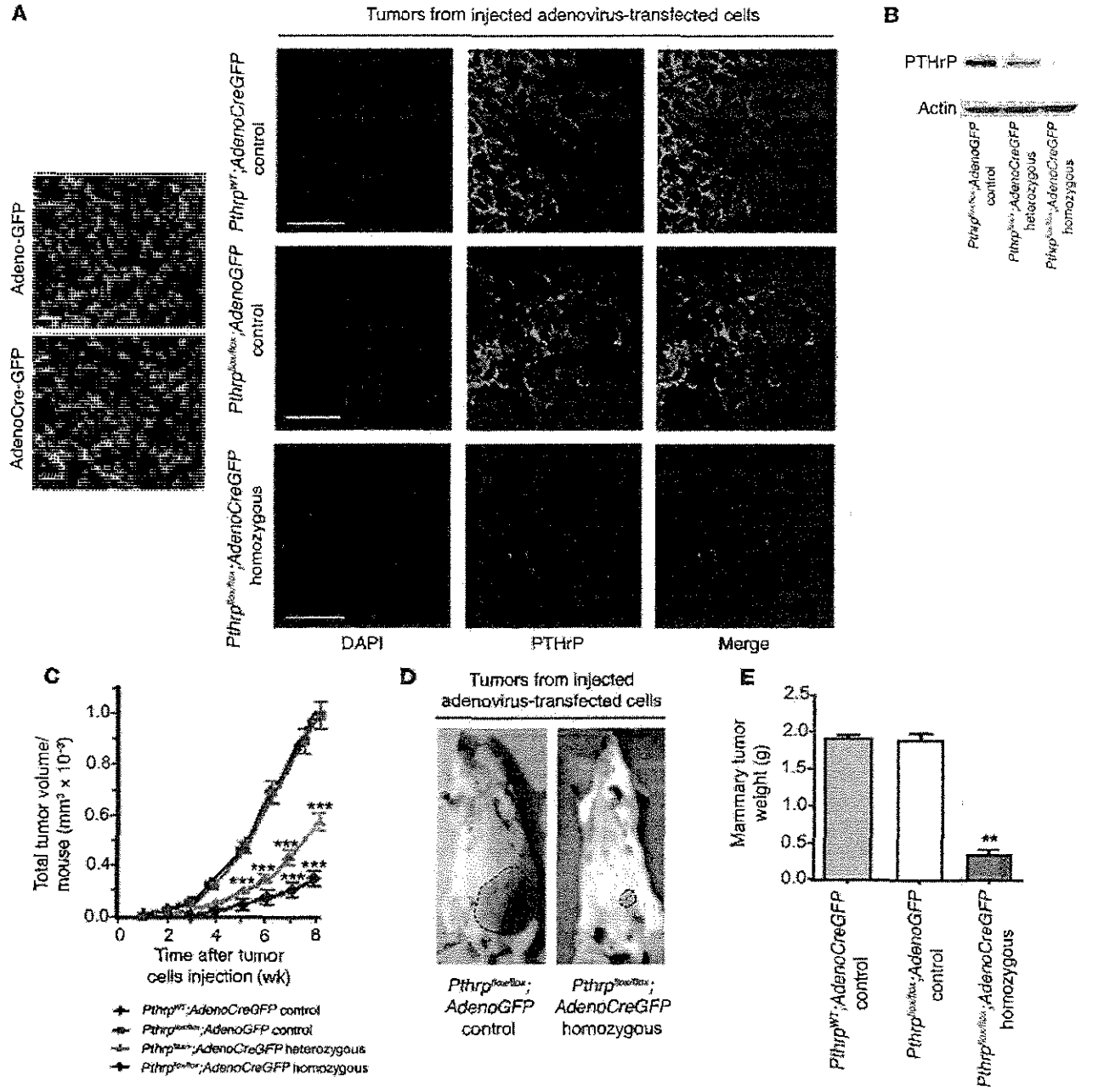

Figure 4

A more complete ablation of Pthrp by Cre-carrying adenovirus further delays breast cancer initiation and progression. (**A**) Adenovirus-transfected tumor cells selected by flow cytometry: left, cell fluorescence for GFP; right, confocal images of IF staining for DAPI (blue) and PTHrP (green) in mammary tumors derived from injected adenovirus-transfected tumor cells illustrating near-complete disappearance of PTHrP expression in homozygous tumors. Scale bars: 200 μm. (**B**) Western blot quantifying PTHrP expression in *Pthrp*^{floxflox} tumor cells transfected with adenoGFP. Lane 1, control, *Pthrp*^{floxflox} adenoGFP; lanes 2 and 3, hetero- and homozygous, *Pthrp*$^{floxl+}$ or *Pthrp*^{floxflox} adenoCreGFP, respectively. (**C**) Tumor volume per animal for tumors derived from adenovirus-transfected tumor cells injected into the MFPs of syngeneic mice. Values represent mean ± SD, $n = 12$ mice for each group. ***$P < 0.001$. (**D**) Tumor load in whole animals 8 weeks after adenovirus-transfected cell injection. (**E**) Average weight of breast tumor load per mouse at sacrifice. Values represent mean ± SD, $n = 12$ mice per group. **$P < 0.01$.

ing high PTHrP levels presented higher rates of metastasis and increased or earlier mortality (40–43). A recent report reached opposite conclusions, but relied on animal models with a very late-onset oncogenic system (*neu* based), which is more relevant to tumors arising in older animals (44), and used mice with a heterogeneous genetic background. The age at which oncogenic induction occurs and the host's genetic background are 2 factors that significantly affect the biological behavior of tumors (45, 46) and likely account for the

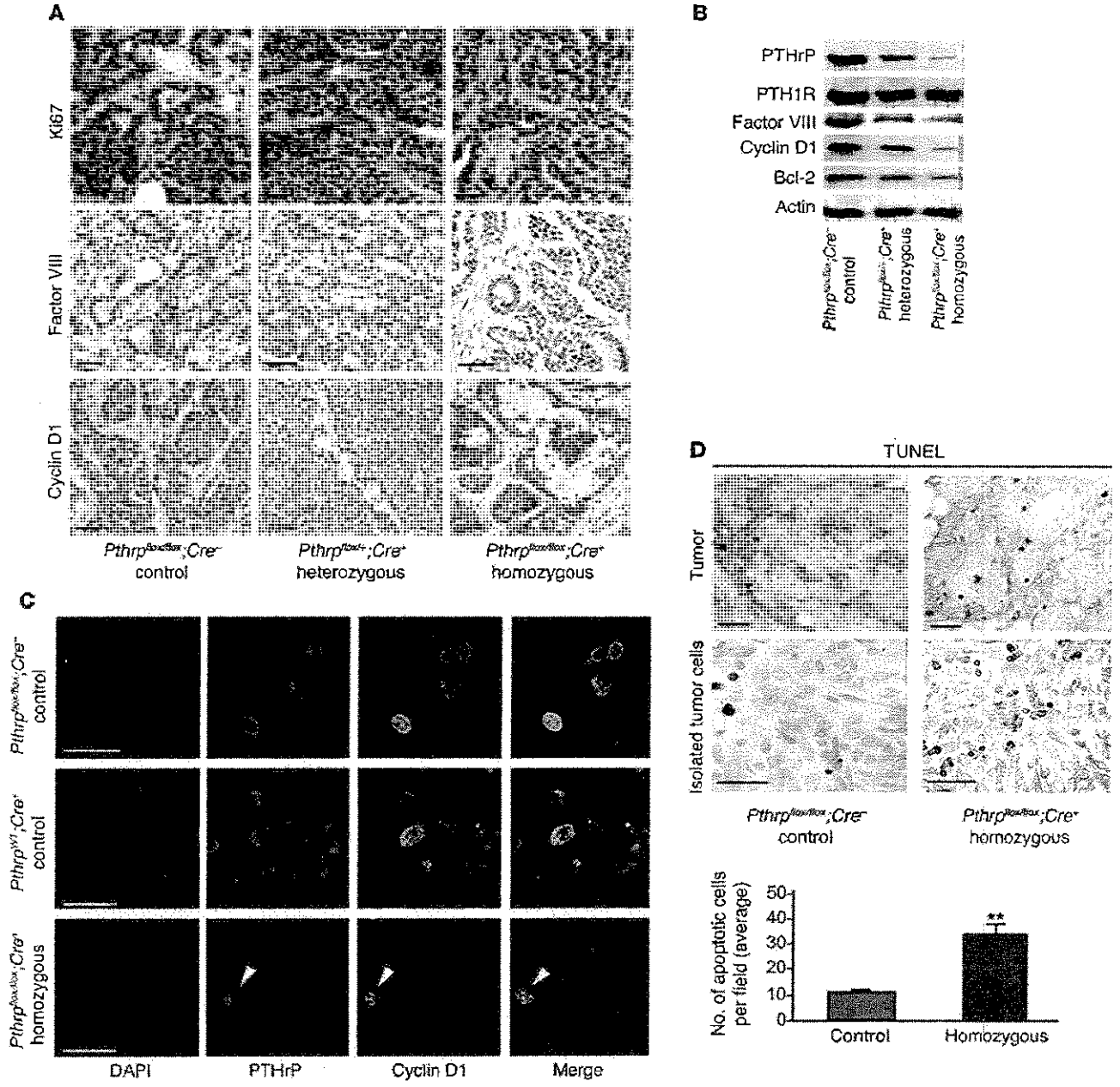

Figure 5

Pthrp ablation modifies cell-cycle, apoptosis, and angiogenesis events. (**A**) IHC staining of tumor tissues at 13 weeks showing a decrease in differentiation factor Ki67 (top), angiogenesis factor VIII (middle), and cyclin D1 (bottom) with degree of *Pthrp* ablation. (**B**) Western blot illustrating no change in PTH/PTHrP receptor 1 expression, but decreases in factor VIII, cyclin D1, and Bcl-2 with degree of *Pthrp* ablation. (**C**) Confocal images of IF staining in cultured cells isolated from tumors showing colocalization of PTHrP and cyclin D1 expression. The residual cells that escaped ablation and are still capable of expressing PTHrP are the only ones expressing cyclin D1 (homozygous, bottom row, arrowheads). Shown are DAPI (blue), PTHrP (red), and cyclin D1 (green). (**D**) TUNEL staining of breast tumor tissue (top) or in cells isolated from tumors and cultured (bottom), showing more abundant apoptotic events in homozygous tumors. Bottom panel: histogram showing average number of apoptotic cells per field in isolated tumor cells. Scale bars: 50 μm. Values represent mean ± SD. $^{**}P < 0.01$.

discrepancy observed with our results. Clinical studies reporting a good prognostic value for PTHrP in breast cancer have concentrated on cohorts in which neo-adjuvant cases were eliminated, therefore focusing on the less aggressive cases (47). In contrast, our animal model is more relevant to early-onset, pregnancy-independent, highly aggressive breast tumorigenesis, corresponding to a category of patients likely to require neo-adjuvant treatment.

In our mouse model, *Pthrp* ablation was accompanied by a near-complete disappearance of the CXCR4 receptor, a member of the chemokine superfamily that regulates cell migration and target-

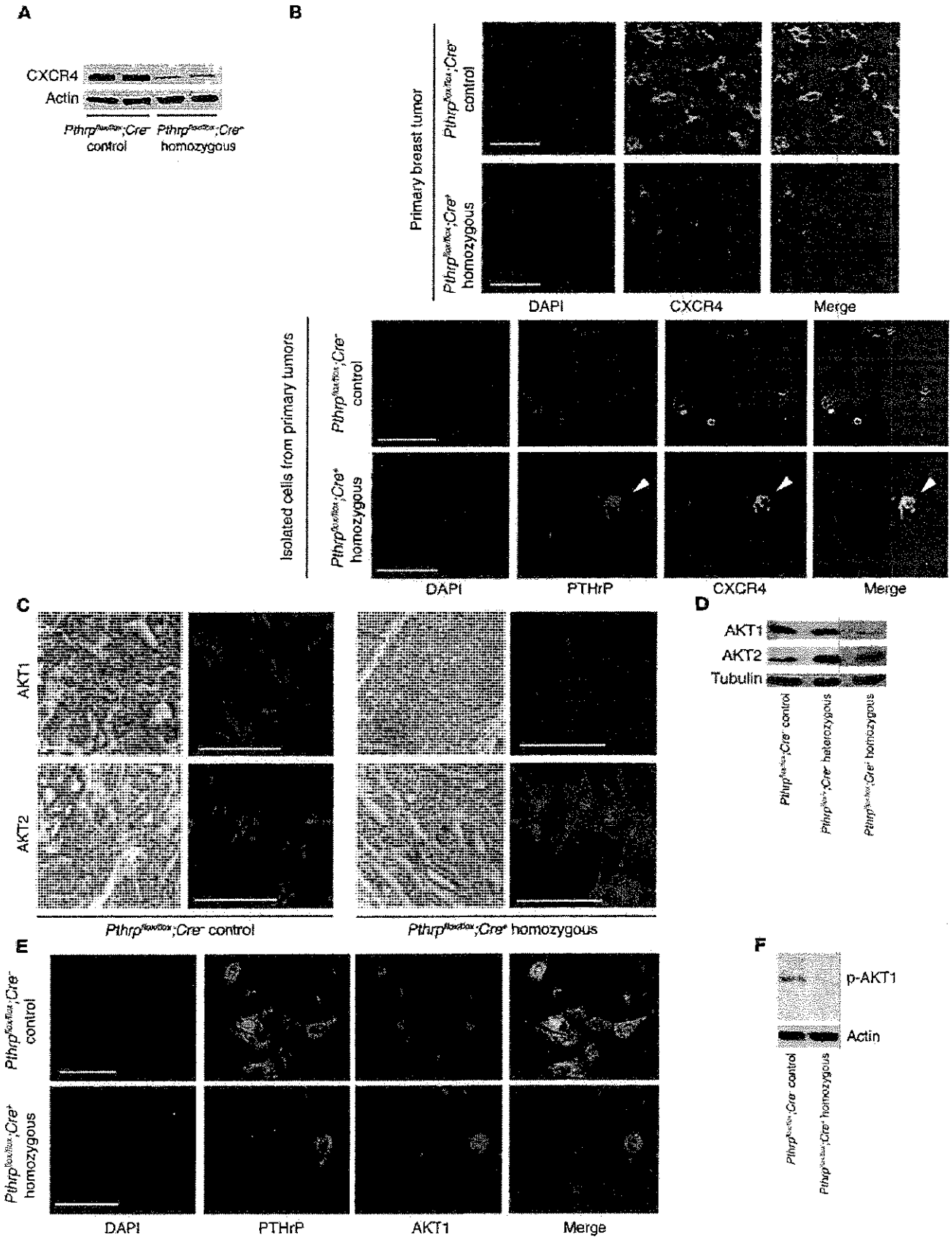
A
CXCR4
Actin
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre- homozygous
B
Primary breast tumor
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous
DAPI
CXCR4
Merge
Isolated cells from primary tumors
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous
DAPI
PTHrP
CXCR4
Merge
C
AKT1
AKT2
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous
D
AKT1
AKT2
Tubulin
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ heterozygous
Pthrp flox/flox;Cre+ homozygous
E
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous
DAPI
PTHrP
AKT1
Merge
F
p-AKT1
Actin
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous

Figure 6
PTHrP is involved in CXCR4 and AKT expression control. (A) Western blot showing decreased CXCR4 expression in homozygous tumors. (B) Confocal images of IF. Top: primary breast tumors (control 13 weeks, homozygous mice 18 weeks). Bottom: cells isolated from these tumors and cultured. CXCR4 expression is significantly reduced with *Pthrp* ablation. Residual cells that escaped *Pthrp* ablation and are still expressing PTHrP are the only ones expressing CXCR4 (arrowheads). (C) IHC (left) and IF (right) images for AKT1 and AKT2 in tumors from control and ablated mice. Shown are DAPI (blue), AKT1 (top, red), and AKT2 (bottom, red). (D) Western blot showing decrease in AKT1 and increase in AKT2 concurrent with *Pthrp* ablation. (E) Confocal images of IF staining of cultured cells from control (top) and homozygous (bottom) tumors. The residual cells that escaped ablation and are still expressing PTHrP also express AKT1, although a small level of AKT1 is detectable in PTHrP-negative cells. Shown are DAPI (blue), PTHrP (green), and AKT1 (red). (F) Western blot of tumor extracts for AKT1 Ser473 phosphorylation. Scale bars: 50 μm.

ing organisms (48, 49). Tumor cells, including those from breast cancer, produce chemokine receptors that promote adhesion to specific cells presenting the appropriate surface ligand (the known ligand for CXCR4 is CXCL12). Organs with the highest expression of CXCL12 correlate with the most common breast cancer metastasis sites (50). The consequence is not only adhesion of the tumor cell to the ligand-expressing site, but acrin polymerization and pseudopodia formation, resulting in enhanced invasiveness (48, 49). The CXCR4/CXCL12 combination is the most important chemokine mechanism regulating metastatic potential to the bone marrow, lungs, liver, and brain (49, 50). CXCR4 has been identified as a signature gene in primary breast cancers expressing the lung and the bone metastasis signature (51, 52); its expression in breast tumors increases with cancer progression (53) and correlates with poor survival rates (54). A study of breast cancer patients who developed skeletal metastases confirmed a generalized elevated expression for both PTHrP and CXCR4 (55).

In breast and prostate cancer cells, the CXCR4 receptor/CXCL12 ligand interaction results in activation of the PI3K/AKT pathway, which drives cell growth and survival (56) and is a key signaling route for CXCR4 (50, 57). The PI3K/AKT pathway is also essential to the transmission of oncogenic signaling from the middle T oncoprotein and to development of mammary adenocarcinomas in the PyMT system (39, 58). Here, *Pthrp* ablation and consequent CXCR4 depletion were accompanied by a significant decrease in total levels of the prosurvival AKT1 isoform and by an increase in the AKT2 isoform. AKT2 ablation accelerates tumor induction, and the 2 AKT isoforms possess opposite oncogenic effects (59). In striking similariry with our observations, a knockout of *Akt1* interferes with mammary tumor initiation and growth in PyMT-MMTV mice, without hindrance to mammary development (59), but it is difficult to speculate whether PTHrP acts through acute or adaptive effect on AKT and CXCR4 signaling. *Pthrp* ablation has repercussions on the cell cycle (G_0/G_1 to S transition, cyclin D1, and Ki67) and enhances apoprotic events. Similarly, in the PyMT-*Akt1* knockout mice, the delay in tumorigenesis is related to inhibition of cell proliferation, to a decrease in Ki67 and cyclin D1, and to promotion of apoptosis (59). Interestingly, cotargeting *Pthrp* and *Akt1* by gene ablation and siRNA techniques provides a stronger inhibition of proliferation over either blockade alone (Figure 7, A–B), suggesting the existence of PTHrP-independent signaling through AKT. This is supported by the presence of residual AKT1 in *Pthrp*-ablated cells (Figure 6E) and suggests that a combined therapeutic approach could provide enhanced efficacy.

The CXCR4/CXCL12 interaction drives metastatic progression by activation of angiogenesis, chemotaxis, adhesion, and invasion processes (50), and neutralization of CXCR4/CXCL12 action in vivo by antibodies (48), inhibitory peptide (60), or siRNA (61) attenuates metastases in mouse models. Here, *Pthrp*-targeted ablation also reduces the number of tumor cells in peripheral blood and bone marrow and delays metastasis in lungs, the preferred metastatic site in the PyMT-MMTV mouse. Importantly, the merastatic tumors that eventually develop in homozygous animals present a pattern of positive expression for both PTHrP and CXCR4, indicating that it is mainly the tumor cells that escaped *Pthrp* ablation that are selected to create the invasive population that develops into metastases. Since almost all residual expression of PTHrP and CXCR4 in homozygous primary tumors colocalizes to the same cells, PTHrP appears to be an important control for CXCR4 expression, with consequences to survival and metastasis. An involvement of PTH in CXCL12 production by osteoblasts (62) reinforces the concept that as a member of the PTH family, PTHrP plays a role in CXCR4/CXCL12 signaling.

Previous work with anri-PTHrP antibodies revealed inhibition of bone turnover and osteolytic metastases (63, 64). Here, treatment of nude mice bearing PTHrP-positive human breast cancer

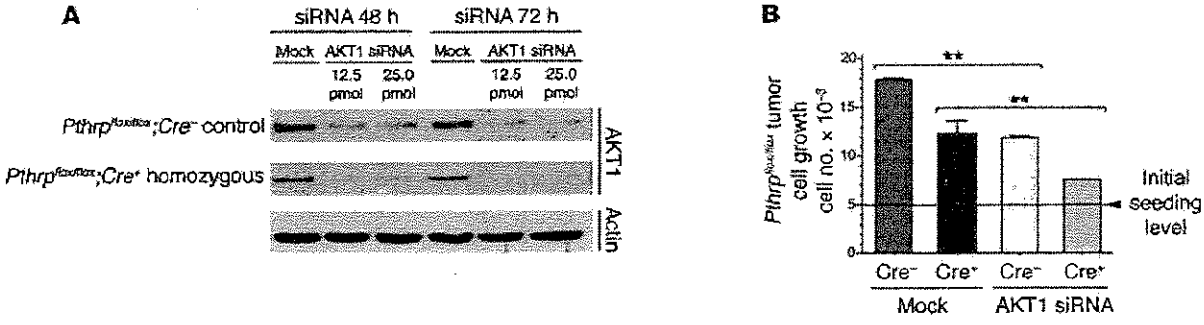

Figure 7
AKT1 inhibition by siRNA enhances Pthrp ablation effect on tumor cell growth inhibition. (A) Western blots for AKT1 expression in control (top) and homozygous cells (bottom) transfected with AKT1 siRNA or mock sequence (48 or 72 hours). (B) Proliferation of isolated $Pthrp^{flox/flox}$ tumor cells from control (Cre^-) or homozygous (Cre^+) mice after AKT1 siRNA or mock transfection (72 hours). Representative experiment out of 3 replicates. $^{**}P < 0.05$ for all except Cre^- mock versus Cre^+ siRNA: $P < 0.0001$. Error bars represent SD.

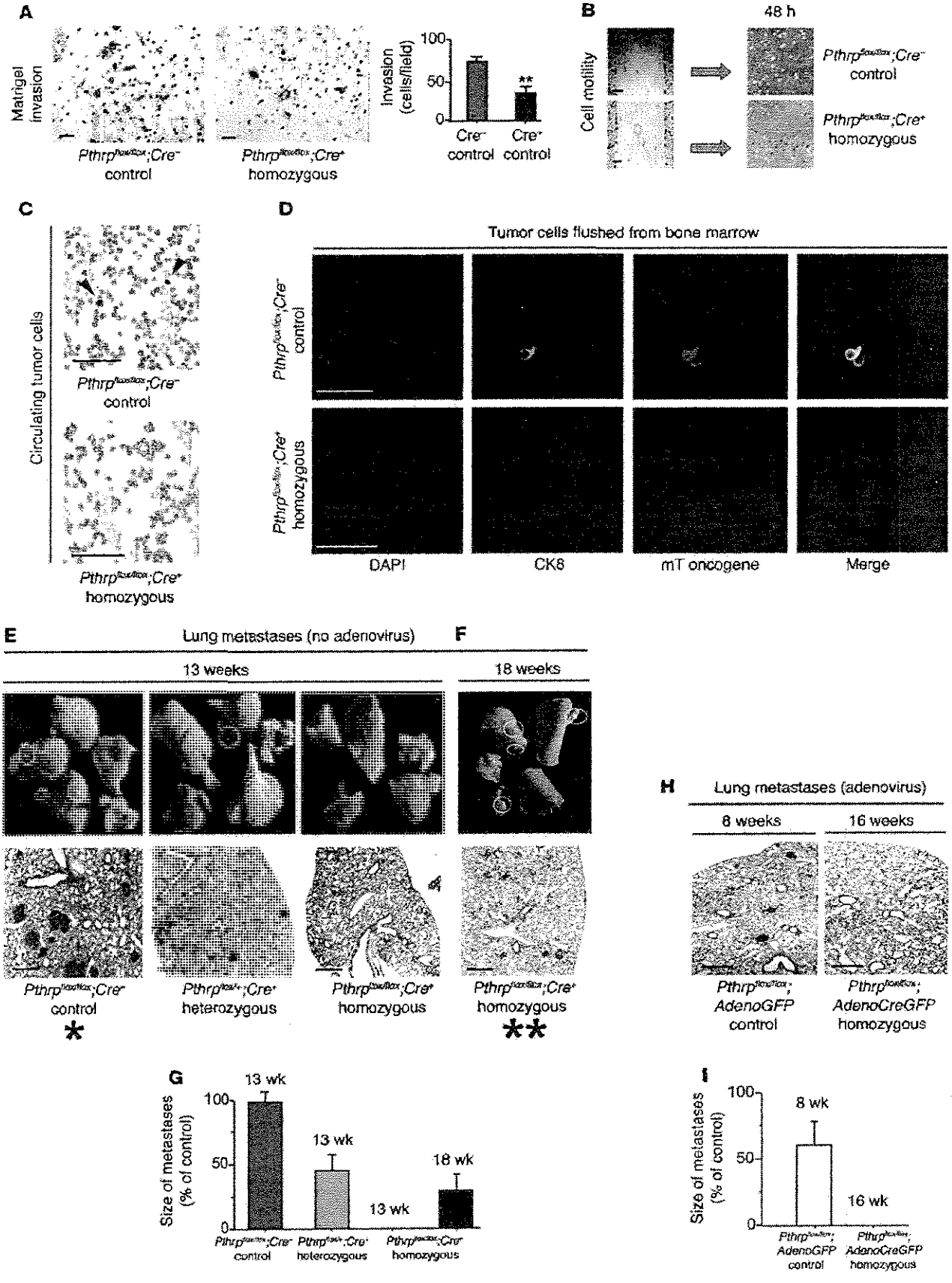
A
Matrigel invasion
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous
Invasion (cells/field)
100
50
0
**
Cre- control
Cre+ control
B
48 h
Cell motility
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous
C
Circulating tumor cells
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous
D
Tumor cells flushed from bone marrow
Pthrp flox/flox;Cre- control
Pthrp flox/flox;Cre+ homozygous
DAPI
CK8
mT oncogene
Merge
E
Lung metastases (no adenovirus)
13 weeks
F
18 weeks
Pthrp flox/flox;Cre- control
*
Pthrp flox/+;Cre+ heterozygous
Pthrp flox/flox;Cre+ homozygous
Pthrp flox/flox;Cre+ homozygous
**
H
Lung metastases (adenovirus)
8 weeks
16 weeks
Pthrp flox/flox; AdenoGFP control
Pthrp flox/flox; AdenoCreGFP homozygous
G
Size of metastases (% of control)
100
50
0
13 wk
13 wk
13 wk
18 wk
Pthrp flox/flox;Cre- control
Pthrp flox/+;Cre+ heterozygous
Pthrp flox/flox;Cre+ homozygous
I
Size of metastases (% of control)
100
50
0
8 wk
16 wk
Pthrp flox/flox; AdenoGFP control
Pthrp flox/flox; AdenoCreGFP homozygous

Figure 8
PTHrP drives metastatic spread. (**A**) Matrigel growth of tumor cells from *Pthrp*$^{loxl/lox}$;*Cre*$^{-}$ or *Pthrp*$^{lox/flox}$;*Cre*$^{+}$ tumors and histogram showing reduced invasive capacity for ablated cells (22 hours). $^{**}P < 0.01$. (**B**) Cell motility test after wounding (48 hours); *Pthrp*-ablated cells show slower motility than control cells. (**C**) Epimet stain of cytospins for detection of circulating tumor cells (arrowheads show pan-cytokeratin–positive cells, 18 weeks). No tumor cells are detectable in blood of homozygous mice. (**D**) Tumor cells flushed from bone marrow (IF stain: cytokeratin 8/PyMT double positives) in control animals only. (**E**) Lung metastases are slower to appear in heterozygous mice. (**F** and **G**) Lung metastases appear in homozygous mice even later (between 13 and 18 weeks). (**H** and **I**) Lung metastases after MFP injection of adenovirus-transfected tumor cells. Lung metastases appear earlier in control mice than in the spontaneous tumor model (**E** and **G**), but are not detectable in homozygous ablated mice (adenovirus transfected) at 16 weeks. (All groups for **E** to **I**, $n = 9$). Scale bars: 100 μm (**A**–**C**); 50 μm (**D**); 200 μm (**E**, **F**, and **H**). Error bars represent SD. Large single and double asterisks refer to corresponding stages in Figure 9.

xenografts with anti-PTHrP mAbs reduced both primary growth and lung metastatic tumor development, which suggests a PTHrP role distinct from bone turnover control and calcium homeostasis. Overall, our data demonstrate that in a model of early-onset, pregnancy-independent, highly aggressive breast tumorigenesis, PTHrP plays a tumor-promoting role in initiation, progression, and metastasis, in part through upregulation of the CXCR4 and AKT pathways (Figure 11). Autocrine, intracrine, and paracrine effects have been reported for PTHrP (8, 27, 65) and are likely involved in its pleiotropic cancer-promoting activities, since the PTH1 receptor is present throughout the tumor tissue and is unaffected by *Pthrp* ablation. In carcinomas, there is frequent alteration in the expression of many growth factors involved as autocrine and paracrine mediators of stromal-epithelial interactions (66). Our results support the idea that some PTHrP-mediated effects observed in vivo cannot be observed in vitro, which points to important tumor/stromal interactions that must be considered when examining PTHrP functions during breast cancer progression. Finally, the effective inhibition of PTHrP action in human cancer xenografts by our neutralizing antibodies constitutes a proof of principle for therapeutic consideration.

Methods

Conditional ablation of the Pthrp gene in mouse ME. C57BL/6 *Pthrp* floxed mice (*Pthrp*$^{flox/flox}$) (38) were backcrossed 9 generations to FVB animals. Marker analysis (Jackson Laboratory) confirmed a more than 99% FVB/NJ background. Crossing these mice with PyMT-MMTV strain PyMT 634 (34) or MMTV-Cre (35) mice (FVB background, Cre7 type) produced PyMT-MMTV;*Pthrp*$^{flox/flox}$ and PyMT-MMTV;*Pthrp*$^{flox/+}$ and MMTV-*Cre*;*Pthrp*$^{flox/flox}$ and MMTV-*Cre*;*Pthrp*$^{flox/+}$ mice. Crossing these animals produced PyMT-MMTV;*Pthrp*$^{flox/flox}$;*Cre*$^{+}$ (homozygous), PyMT-MMTV;*Pthrp*$^{flox/+}$;*Cre*$^{+}$ (heterozygous), PyMT-MMTV; and *Pthrp*$^{flox/flox}$;*Cre*$^{-}$ or PyMT-MMTV;*Pthrp*$^{flox/+}$;*Cre*$^{-}$ (controls) mice.

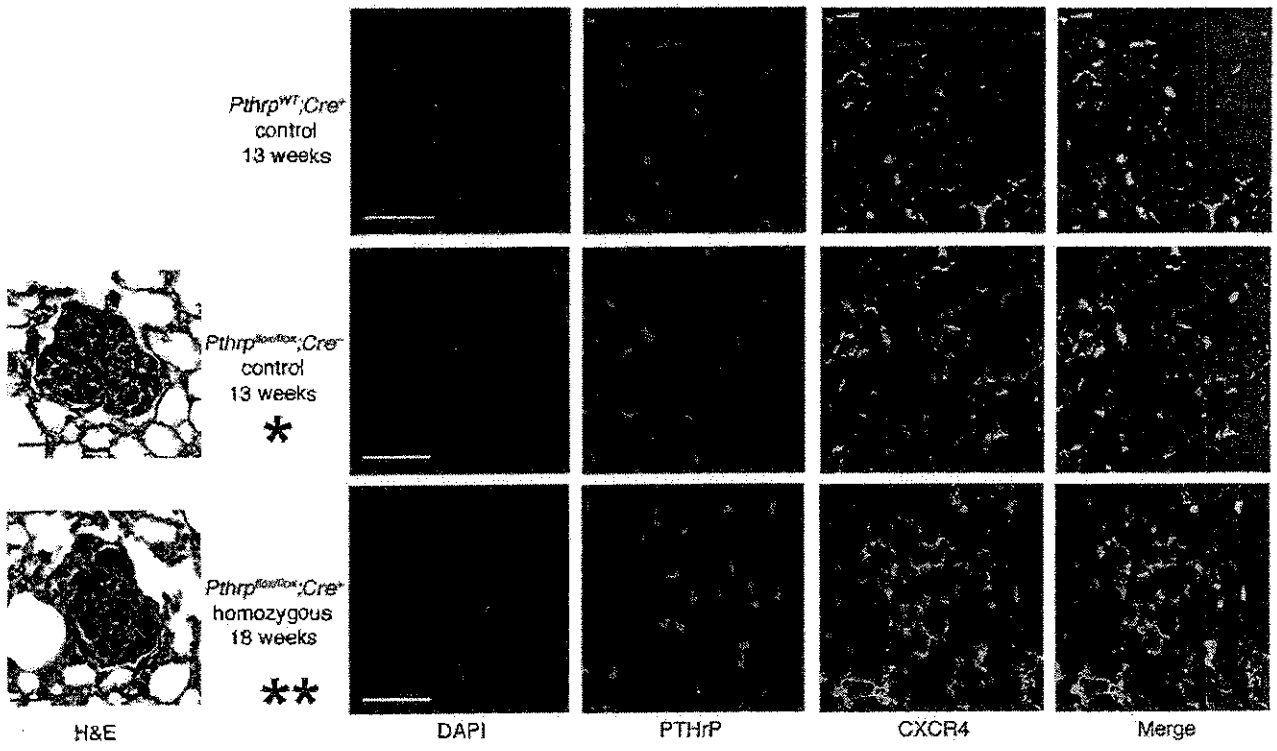

Figure 9
Spontaneous lung metastases are PTHrP and CXCR4 positive. H&E stain (left) and IF confocal (right) of spontaneous lung metastases (no adenovirus) at same-size tumor (control 13 weeks, homozygous 18 weeks); lung metastases in homozygous mice are PTHrP and CXCR4 positive. Shown are DAPI (blue), CXCR4 (green), and PTHrP (red). Large single and double asterisks indicate corresponding stages illustrated in Figure 8E. Scale bars: 50 μm.

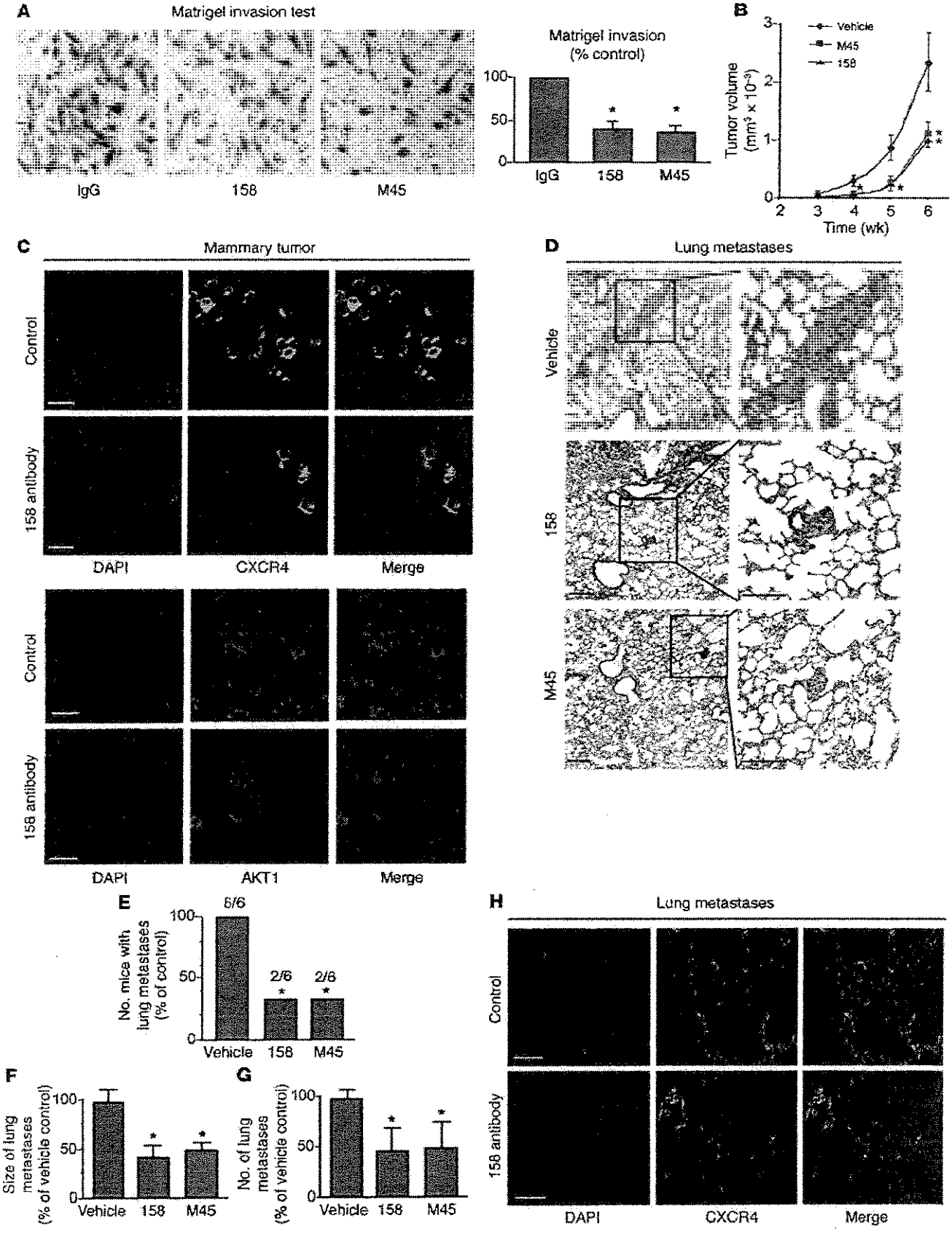
A
Matrigel invasion test
IgG
158
M45
Matrigel invasion
(% control)
100
50
0
IgG
158
M45
B
Vehicle
M45
158
Tumor volume
(mm³ x 10⁻⁸)
3
2
1
0
2
3
4
5
6
Time (wk)
C
Mammary tumor
Control
158 antibody
DAPI
CXCR4
Merge
Control
158 antibody
DAPI
AKT1
Merge
D
Lung metastases
Vehicle
158
M45
E
No. mice with lung metastases (% of control)
100
50
0
6/6
2/6
2/6
Vehicle
158
M45
F
Size of lung metastases (% of vehicle control)
100
50
0
Vehicle
158
M45
G
No. of lung metastases (% of vehicle control)
100
50
0
Vehicle
158
M45
H
Lung metastases
Control
158 antibody
DAPI
CXCR4
Merge

Figure 10
Anti-PTHrP neutralizing mAbs inhibit breast cancer progression in vitro and in vivo. (**A**) Proliferation in Matrigel (24 hours) of human MDA-MB-435 breast cancer cells showing growth-inhibition effect of neutralizing antibodies 158 and M45 in vitro.*$P < 0.05$. (**B**) Tumor volume over time after injection of MDA-MB-435 cells into the MFPs of BALB/c nu/nu mice and treatment with anti-PTHrP mAbs, showing the tumor-reducing effect in vivo. Data are expressed as means of 8 mice in each group. *$P < 0.05$; **$P < 0.01$. (**C**) IF confocal images of mammary tumors 6 weeks after injection of MDA-MB-435 cells in MFPs of nude mice showing decrease of CXCR4 (top panels) and AKT1 (bottom panels) in treated animals. Shown are DAPI (blue), CXCR4 (green), and AKT1 (red). (**D**) H&E staining of lung metastases 6 weeks after injection of MDA-MB-435 in MFPs. Treatment with anti-PTHrP mAbs reduces the size and numbers of lung metastases. (**E–G**) Fewer mice present lung metastases after treatment with either mAb, and the metastases are smaller and fewer in numbers in treated animals. Mean ± SEM. *$P < 0.05$ (**E**); *$P = 0.013$ (**F**); *$P = 0.045$ (**G**). (**H**) IF confocal images of lung metastases in nude mice injected with MDA-MB-435 cells treated (6 weeks) or not with anti-PTHrP mAbs. Lung metastases are CXCR4 positive irrespective of treatment. Shown are DAPI (blue) and CXCR4 (green). Scale bars: 100 μm (**A**); 50 μm (**C** and **H**); 200 μm (**D**).

Immunohistochemistry and IF. Antibodies used were as follows: mouse monoclonal N-terminal anti-PTHrP antibody (1-34, AE-0502, IDS), mouse monoclonal PTH1R antibody (Upstate), mouse monoclonal anti-Cre recombinase and anti–phospho-Akt1 (Ser473) (Abcam), goat anti–total Akt1, Akt2, and Ki67, mouse cyclin D1, Bcl-2, actin, and rabbit polyclonal antibodies for the PTHR1 receptor, $PTHrP_{41-139}$ C-terminal end, tubulin, anti-rabbit and anti-mouse peroxidase conjugates (Santa Cruz Biotechnology Inc.), Alexa Fluor 555–conjugated goat anti-rabbit IgG and Alexa Fluor 488–conjugated goat anti-mouse IgG (Invitrogen), guinea pig anti-mouse cytokeratin 8, donkey anti-guinea pig IgG CY3-labeled, and rabbit anti-mouse cytokeratin 5 (PROGEN Biotechnik GmbH).

Tumors and other tissues were fixed, embedded, sliced, and stained with H&E according to standard protocols. The SK-4100 kit (Victor) was used for immunohistochemistry (IHC), and slides were reacted for diaminobenzidine and analyzed with a Leica DMR microscope and BIOQUANT Nova Prime software (Bioquant). IF staining was conducted on deparaffinized tissue sections or cells fixed on slides. Results were analyzed with an LSM 510 Meta confocal microscope (Carl Zeiss Microimaging).

Whole-mount staining. MFPs were removed, spread on a glass slide, air dried, fixed, delipidated, and stained with 0.5% Neutral Red (Sigma-Aldrich).

Western blotting. Proteins were extracted from tissues, and samples (30–50 μg) were fractionated by SDS-PAGE electrophoresis, transferred to PVDF membranes, reacted with primary and secondary antibodies, and developed by enhanced chemiluminescence according to standard methods. Whole mammary glands were prepared according to Ackler et al. (67).

Culture of mouse breast tumor cells from primary tumors. Tumors were harvested, minced, and incubated in 2.4 mg/ml collagenase B and dispase II (Roche) in DMEM (no FBS) at 37°C for 2 hours. Floating cells were washed, pelleted, resuspended, and propagated in complete DMEM.

Adenovirus vector. Tumor cells in culture were infected with a recombinant adenovirus vector (10^8 viral particles/ml) containing a GFP-Cre transgene for the Cre recombinase. Forty-eight hours later, cells were selected on a BD FACSAria II cell sorter. GFP-positive cells (1×10^6 cells) were injected into the fourth MFP of anesthetized 5-week-old syngeneic FVB mice (Charles River).

Cell-cycle analysis, PTHrP, and calcium assays. Cultured cells were stained with propidium iodide and analyzed for cell cycle by flow cytometry (FACSCalibur). Levels of PTHrP in culture medium or serum were measured by 2-site immunoradiometric assay (Beckman-Coulter). Serum calcium was determined with a Synchron 67 Autoanalyzer (Beckman).

siRNA transfection for Akt1 knockdown. Cells isolated from mammary tumors were transfected with siRNA *Akt1* targeting sequences (s62216) as follows: sense: CUCAAGUACUCAUUCCAGAtt, antisense: UCUGGAAUGAGUACUUGAGgg (bases in lower case are overhangs not corresponding to the original target sequence), and negative control (Ambion). Cell proliferation was assayed by hemocytometer counting.

Tumor cell detection in peripheral blood and in bone marrow. Blood was collected from sacrificed animals by cardiac puncture. Cytospin-concentrated samples were stained with the Epimet Epithelial Cell Kit (Micromet). Bone marrow flushed with PBS from tibias and femurs cut at the knee joint was tritrated through a 25-gauge needle. Samples (2 tibias and 2 femurs/animal) were counted with a hemocytometer and spread onto Cytospin Immunoselect Adhesion Slides (Squarix). Tumor cells were detected with pan-cytokeratin mAb A45-B/B3 or cultured in DMEM with 10% FBS.

Matrigel invasion and wound-healing assays. MDA-MB-435 cells were tested for invasion in Matrigel for 20–24 hours with or without mAbs or IgG (10 μg/ml) according to standard methods. Invading cells were counted after paraformaldehyde fixation and hematoxylin staining. For motility assays, cells were grown on poly-D-lysine–coated plates (Millipore). The cell carpet was scraped and the growth of cells into the scraped area monitored.

Lung metastases measurements. Photomicrographs of H&E-stained slides of lung metastases were analyzed with ImageJ software (http://rsbweb.nih.gov/ij/index.html) to assay metastatic surface/lung ($n = 9$ mice).

Anti-$PTHrP_{(1-33)}$ mAbs. We generated 2 PTHrP-specific mAbs against $hPTHrP_{1-33}$ peptide; hybridomas 158 and M45 produced mAbs (subclasses IgG3 for mAb 158 and IgMκ for mAb M45) with strong binding to $hPTHrP_{1-33}$. The mAbs were highly specific (no reaction with PTH), and no cross-reactivity between antibodies and other fragments of PTHrP was observed. The 158 and M45 hybridomas have been deposited at the International Depositary Authority of Canada (accession numbers 060808-02 and 060808-01).

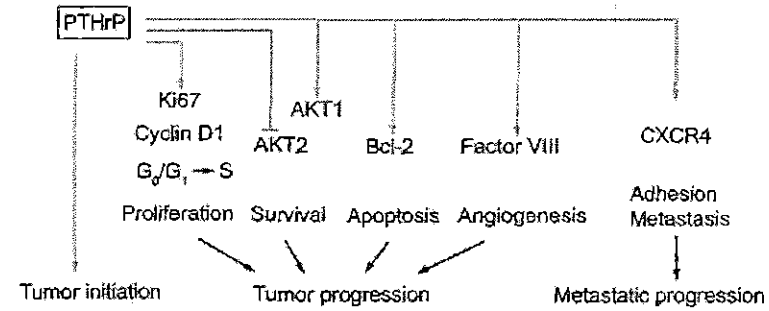

Figure 11
PTHrP influences several key steps in breast cancer. Interactions are described here for PTHrP in tumor cell proliferation; through its effects on cell proliferation factor Ki67, cell-cycle progression regulator cyclin D1, and the G_0/G_1 to S transition, PTHrP is involved in very early steps of oncogenesis. PTHrP influences breast tumor cell survival, apoptosis, and angiogenesis through control of levels of expression for crucial signaling molecules such as AKT1/AKT2, Bcl-2, and factor VIII. Of great interest is the observation that PTHrP is involved in the control of CXCR4 expression and consequently also plays a role in metastatic spread.

Treatment of nude mice with anti-PTHrP mAbs. 1×10^6 human cancer cells (MDA-MB-435) were injected into the fourth MFP of 4- to 5-week-old BALB/C nu/nu females (Charles River). Starting the next day, antibodies were injected subcutaneously (200 μg) every 48 hours for 6 weeks. Mouse IgG_3 isotype control was from R&D Systems. Primary tumor growth rates were determined by plotting the means of 2 orthogonal diameters of the tumor measured at 5-day intervals. Tumor volume was measured once weekly. Animals were sacrificed 6 weeks after tumor implantation.

PyMT-MMTV animal handling. Tumor-bearing animals were examined by palpation twice a week until they were 13 weeks of age. Mice with excessive tumor burden (> 1.5 cm) were euthanized. For Kaplan-Meier, animals were sacrificed when total tumor load exceeded 20% of animal weight. All experiments were carried out in compliance with regulations of the McGill University Institutional Animal Care Committee. All animal surgeries were conducted in accordance with principles and procedures dictated by the highest standards of humane animal care.

Statistical analysis of tumor progression and tumor growth. Numerical data are presented as the mean ± SD. The data were analyzed by ANOVA followed by a Bonferroni's post-test to determine the statistical significance of differences. All statistical analyses were performed using Instat Software (GraphPad Software), and $P < 0.05$ was considered statistically significant. Specific tests are mentioned in the text.

Study approval. These animal studies were approved by the McGill University Animal Compliance Office.

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Parathyroid Hormone Related Protein (PTHrP) in Tumor Progression*

12

Richard Kremer, Jiarong Li, Anne Camirand, and Andrew C. Karaplis

Abstract

Parathyroid hormone-related protein (PTHrP) is widely expressed in fetal and adult tissues and is a key regulator for cellular calcium transport and smooth muscle cell contractility, as well as a crucial control factor in cell proliferation, development and differentiation. PTHrP stimulates or inhibits apoptosis in an autocrine/paracrine and intracrine fashion, and is particularly important for hair follicle and bone development, mammary epithelial development and tooth eruption. PTHrP's dysregulated expression has traditionally been associated with oncogenic pathologies as the major causative agent of malignancy-associated hypercalcemia, but recent evidence revealed a driving role in skeletal metastasis progression. Here, we demonstrate that PTHrP is also closely involved in breast cancer initiation, growth and metastasis through mechanisms separate from its bone turnover action, and we suggest that PTHrP as a facilitator of oncogenes would be a novel target for therapeutic purposes.

12.1 PTHrP Background, Discovery, Gene Sequence and Protein Structure

12.1.1 Background

Malignancy-associated hypercalcemia (MAH) is a well-recognized syndrome that occurs in patients suffering from certain malignant cancers. The classic signs and symptoms of hypercalcemia are confusion, polydipsia, polyuria, constipation, nausea, vomiting and eventually coma. Hypercalcemia associated with malignancy of nonparathyroid tissues frequently occurs during

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bone invasion by tumor cells, where bone resorption is the direct result of osteoclast action. Osteoclastic bone-degrading activity is stimulated by several cytokines including MIP-1α, TNF-β, IL-1β, IL-3 and IL-6, which are produced locally by the tumor cells [1–4]. MAH is commonly associated with malignant tumors of the breast, lung, head and neck, esophagus, cervix, skin or kidney [5].

In 1936, Gutman conducted the first large group study of MAH; these patients suffered from myeloma and breast cancer, and most of them presented bone metastases [6]. Fuller Albright in 1941 was the first to propose that a PTH-like humoral factor was responsible for the hypercalcemia in patients with renal carcinoma which resolved after irradiation of bone metastasis. In the following years, the concept was accepted and the term "ectopic PTH syndrome" became widely used to describe patients with high circulating plasma calcium concentrations, low phosphorus, and few or no bone metastases [7–9]. In 1969, a thorough review was made of cases where patients suffered symptoms of hyperparathyroidism yet exhibited malignant tumors of nonparathyroid origin. The conclusions drawn from this work were that some nonparathyroid tumor cells could initiate transcription and translation of the *PTH* gene and secrete PTH [10]. Such cases of "ectopic PTH syndrome" [11] included kidney adenocarcinoma [12] and a malignant hepatoblastoma [13].

Rare cases were found where nonparathyroid tumors secreted PTH, and many studies demonstrated that the immunological properties of circulating of PTH-like material in hypercalcemic patients with nonparathyroid cancer or primary hyperparathyroidism were distinct from those of the immunoreactive PTH found in the serum of patients with primary hyperparathyroidism [14, 15]. In 1980, Stewart and associates established the first full biochemical characterization for 50 consecutive patients with cancer-associated hypercalcemia, with or without bone metastases. This study delineated characteristic laboratory findings that now define the PTH-like syndrome, and coined the term humoral hypercalcemia of malignancy (HHM): elevated nephrogenous cyclic AMP excretion levels, high serum calcium, low serum phosphorous, marked reduction in 1,25-dihydroxyvitamin D and low or suppressed reactivity with PTH antisera.

12.1.2 Discovery

Based on the biochemical characterization of humoral hypercalcemia of malignancy, much effort was devoted to the problem of identifying and isolating the tumor-secreted nnknown factor that was responsible for this syndrome. In 1987, three independent groups simultaneously achieved: (1) purification of an active component with a molecular weight of 18 kDa from a human lung cancer cell line (BEN) [16], (2) purification of a 6-kDa active component from cultured human renal carcinoma cells [17], and (3) purification of a 17-kDa active component from a human breast tumor biopsy [18]. Most interesting was the fact that the N-terminal amino acid sequence of these adenylate cyclase-stimulating proteins revealed outstanding homology to PTH, identifying the existence of a PTH-like factor in those cancer cells.

Using the partial amino acid sequence information from these discoveries, oligonucleotide probes were synthesized and used to identify complementary DNAs (cDNAs) encoding BEN cell mRNA [19], mRNA from a human renal carcinoma [20] and mRNA from a renal carcinoma cell line [21]. Characterization of those clones revealed a gene and peptide sequence similar to that of human PTH. Eight of the initial 13 residues of the mature protein and the final 2 residues in the signal peptide were identical to those of human PTH. The term parathyroid hormone-related protein (PTHrP) derives from this high homology to the N-terminal sequence of PTH.

The elucidation of the PTHrP sequence allowed investigators to synthesize fragments of the peptide and to study their effects in different experimental conditions. Kemp et al. demonstrated that the N-terminal fragment of PTHrP (1–34) exhibited PTH-like activity in rat and chicken kidney and produced effects commonly seen in HHM such as increased excretion of

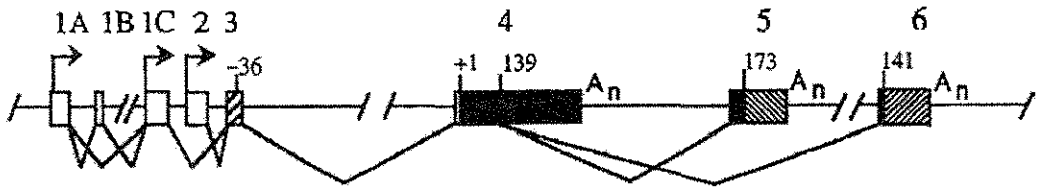

Fig. 12.1 Structure of human parathyroid hormone-related protein (PTHrP) gene (adapted from Philbrick et al. [35])

cAMP and phosphorus, and reduced urinary calcium excretion [22]. Using radioligand binding techniques, Nickols et al. were able to show that amino-terminal fragments of PTH and PTHrP interacted with identical receptors present on rabbit kidney microvessels and tubules [23]. Immunoassays were developed and indicated that PTHrP was circulating at high levels in patients with HHM [24, 25]. These findings provided strong evidence that circulating PTHrP produced by various cancers was the causal agent responsible for hypercalcemia.

12.1.3 Gene Sequence and Protein Structure

The *Pthrp* gene is localized to the short arm of chromosome 12 in humans [26] whereas the *PTH* gene is on the short arm of chromosome 11 [27]. The localization of *PTH* and *Pthrp* on these chromosomes and the similarity in sequence and organization of the two genes provide indirect evidence for a common evolutionary origin [26, 28].

The human *Pthrp* gene (Fig. 12.1) is a complex transcriptional unit which spans more than 15 kb of DNA, with 9 exons and at least 3 promoters. Alternative splicing of the premessenger RNAs produces three PTHrP isoforms differing at their carboxyl-terminal ends and containing 139, 141 or 173 amino acids. Exon 4 encodes a region common to all peptides, while exons 5 and 6 encode the unique carboxy termini of the other two peptides. *PTH* and *Pthrp* genes display an identical pattern of intron/exon organization in the region of exons 2 and 3 [19, 26, 28–30]. In contrast to the human organization, rat and mice *Pthrp* genes are relatively simple with a single promoter homologous to the downstream P3 promoter of the human gene. They produce mature peptides of 141 and 139 amino acids, respectively [31, 32]. The *Pthrp* gene in the chicken yields a single mature peptide of 139 amino acids [33].

The initial translational human PTHrP products undergo complex processing, including separation of the (–36 to –1) pre-pro sequence and endoproteolysis of the full-length (–36 to 139), (–36 to 141) and (–36 to 173) sequence at multibasic sites. All sequences present an N-terminal signal sequence for endoplasmic reticulum entry and a coding 1–139 peptide [34, 35]. The amino acid 35–111 region is dramatically conserved in human, rat, mouse and chicken sequences, with the human and rodent amino acid sequences differing in the 1–111 region by only two residues. This high evolutionary conservation suggests important physiological and biological functions. The 35–111 region is rich in putative proteolytic processing sites, with multiple dibasic amino acid groups [34, 35].

Region 107–111 (-Thr-Arg-Ser-Ala-Trp-) or TRSAW is evolutionally highly conserved, a suggesting that a peptide encompassing this region may be physiologically important [36].

12.2 PTHrP Physiology

12.2.1 PTHrP Functional Domains

Analysis of the *Pthrp* gene sequence reveals several functional domains (Fig. 12.2), including a signal peptide, a PTH-like N-terminal domain, a mid-region domain that begins at amino acid 38 and a unique carboxy-terminal domain named osteostatin [36, 37].

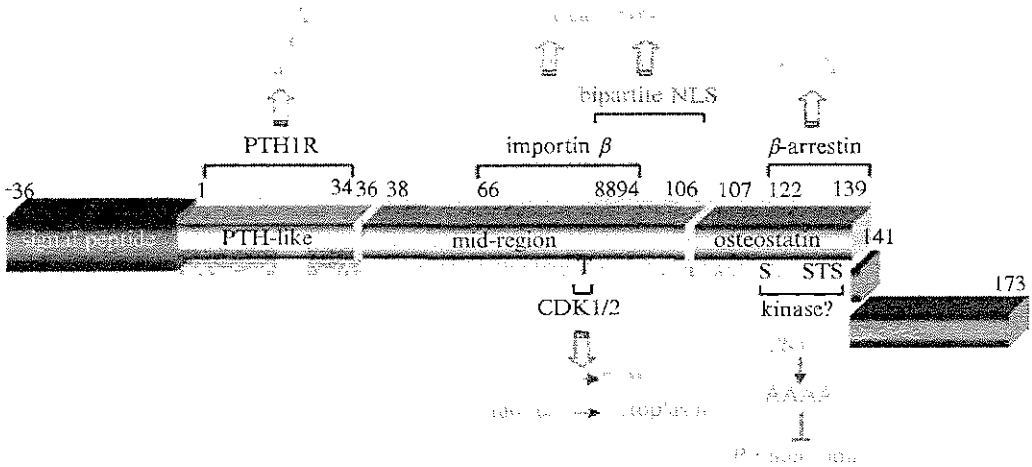

Fig. 12.2 The functional domains of the human PTHrP protein and its interaction partners. T-P denotes phosphorylation of Thr85. SSTS stands for residues Ser119, Ser130, Thr132 and Ser138 and AAAA denotes their replacements by alanines. *CDK* cyclin-dependent kinase, *GPCR* G-protein coupled receptor. *Green bars* show extension of the PTHrP protein in splicing variants –36/141 and –36/173 (adapted from Dittmer [138])

The N-terminal domain of PTHrP (1–36) shares with PTH a homologous sequence that interacts with the PTH/PTHrP type 1 receptor (PTH1R), a class II G-protein coupled receptor (GPCR). Upon binding PTHrP, the PTH1R receptor can either activate the formation of cyclic 3′,5′-adenosine monophosphate (cAMP) by stimulating adenylate cyclase (AC) through Gαs, or activate phospholipase Cβ (PLCβ) through Gαq/11. cAMP then activates protein kinase A (PKA), whereas activated PLCβ stimulates the formation of diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP3). In turn, DAG activates protein kinase C (PKC) and the production of IP3 leading to an increase in the intracellular free Ca^{2+} [38, 39]. The PTH1R receptor can also stimulate the inflnx of extra-cellular Ca^{2+} through regulation of calcium channels [40], and activate protein kinase C through a PLC-independent pathway [41–43]. The PTH1R receptor classical transduction pathways can lead to different biological effects in a cell type-specific manner.

The mid-region PTHrP comprises a bipartite nuclear localization sequence (NLS) consisting of residues 88–91 and 102–106. This NLS sequence may allow PTHrP to accumulate in the nucleus [44–46] and bind rRNA [47, 48]. This peptide likely enters the nucleus immediately after NLS binding to importin-β1 to form a complex that links to the microtubular trackway, where it is pulled by a dynien motor to a nuclear envelope pore and handed over to the pore channel transport machinery [49–52]. Although the NLS exact function is still obscure, it is likely to be of great significance since mice missing the NLS and the C-terminal region of the PTHrP gene present decreased cellular proliferative capacity and increased apoptosis in multiple tissues, retarded growth, early senescence and malnutrition leading to an early death [53]. In addition, in vitro studies indicate that the midregion sequence retains a CDK1/CDK2 phosphorylation site at Thr [85] [54], which suggests that translocation of PTHrP is associated with activation of the cell cycle [44, 55].

The PTHrP C-terminal domain 107–139 was named osteostatin because this peptide inhibits rat osteoclastic bone resorption with an incredibly low EC_{50} value of 10^{-15} M [36, 56, 57].

12.2.2 PTHrP Receptors

PTHrP binds to, and activates, the G-protein coupled receptor (GPCR) for PTH/PTHrP (PTH1R) which is expressed in PTH and PTHrP target cells such as osteoblasts in bone and renal tubular cells. PTH1R in humans and rodents is

encoded by a multi-exon gene with potential for alternate splicing and alternate promoter usage [58]. The *PTH1R* gene is located on chromosome 3 in humans and possesses 14 exons.

PTH1R has seven transmembrane spanning domains [59] and has been cloned from opossum kidney, rat bone, and human bone and kidney [60–62]. The amino terminal region of PTH (1–34) and PTHrP (1–34) interacts with the J-domain, the functional portion of the receptor that contains the seven transmembrane-spanning helices and the connecting loops [63].

Following receptor–agonist interaction, PTH1R is activated and mediates not only the endocrine actions of PTH, but also the autocrine/paracrine actions of PTHrP.

12.2.3 Normal Physiological Functions of PTHrP

PTHrP is widely expressed in fetal and adult tissues, including cartilage, bone, breast, skin, skeletal heart and smooth muscle, uterus and placenta, as well as endocrine organs and the central nervous system [34, 35, 64]. PTHrP is a key regulator for cellular calcium transport and smooth muscle cell contractility, and possesses crucial roles in cell proliferation, development and differentiation [35]. It is important to note that the known biological properties of PTHrP are not only the results of its interaction with PTH1R and its subsequent signal transduction cascades, but also of PTHrP nuclear translocation [65, 66]. PTHrP is known to stimulate or inhibit apoptosis in various settings in a cell- or tissue-specific manner [67–69]. These actions are mostly performed in an autocrine/paracrine and intracrine fashion.

The biological actions of PTHrP are particularly important for bone development during endochondral bone formation. Targeted ablation of *Pthrp* results in homozygous$^{-/-}$ mice dying shortly after birth and presenting abnormalities in endochondral bone development [70]. In contrast, heterozygous *Pthrp*$^{+/-}$ animals are viable but demonstrate a reduction in trabecular bone volume and an early osteoporotic phenotype [71]. In addition, genetic mouse studies indicate PTHrP regulates hair follicle development, mammary epithelial development and tooth eruption [72–76].

12.2.4 PTHrP in Mammary Gland Development

During postnatal breast development in normal mice, PTHrP appears to be expressed by both luminal and myoepithelial cells of the mammary gland [77, 78], while myoepithelial cells and mammary stromal cells express the PTH/PTHrP receptor. Both stromal and myoepithelial cells are important in the branching growth of the mammary gland during sexual maturation and early pregnancy, and PTHrP ligand and receptor are appropriately positioned to participate in this process [77, 79, 80].

Pthrp-null mice die soon after birth of severe musculo-skeletal defects [70], but *Pthrp* rescue in the chondrocytes of these animals leads to a phenotype compatible with life. This strategy generates a mouse PTHrP-sufficient in chondrocytes, but PTHrP-null in all other sites including breast. These mice are characterized by the absence of normal epithelial-mesenchymal signaling cascade, failure to form mammary mesenchyme and the resorption of nascent mammary bud [73, 76].

12.3 PTHrP and Cancer Biology

12.3.1 Breast Cancer

Female breast cancer is a major medical problem with significant public health and societal ramifications. Although breast cancer death rates have declined in recent years, breast cancer remains the most commonly diagnosed cancer and the second leading cause of cancer death in women [81]. Since normal breast growth is regulated by many hormones, growth factors and receptors, it is not surprising that malignant cells derived from breast tissue also express the same hormones, growth factors and receptors. Numerous genes

are controlled by complex regulatory networks and involved in the development and progression of breast cancer, and these genes are the key factors determining the characteristics of each tumor.

12.3.2 PTHrP and Cancer Development

PTHrP was originally discovered in patients with MAH [5]; approximately 80% of hypercalcemic patients with solid tumors have elevated PTHrP plasma concentrations as a result of increased secretion by the tumors [82]. However, PTHrP expression is present in many tumor types even in the absence of hypercalcemia. For instance, the great majority of breast cancer tumor samples show positive staining for PTHrP, and strong *Pthrp* gene activity in breast tumors is associated with onset of bone metastases independent of hypercalcemia [83–87].

Normal prostatic epithelial tissues express low levels of PTHrP, as determined by immunohistochemistry and in situ hybridization. In contrast, overexpression of PTHrP is common in prostate cancer [88, 89], and many prostate cancer cell lines in vitro and metastatic bone lesions in vivo express PTHrP. PTHrP stimulates primary prostate tumor growth and protects cells from apoptotic stimuli [90]. PTHrP expression was found in all major lung cancer cell types [91, 92] and was most common in squamous cell lung cancer [93]. While benign colorectal adenomas and nonneoplastic adjacent mucosal epithelia show no detectable PTHrP expression, about 95% of colorectal adenocarcinomas overexpress PTHrP mRNA and protein, and the expression level is higher in poorly differentiated than in well-differentiated adenocarcinomas [94, 95]. In a clinical study of 76 patients with various hematological malignancies, 50% of the 14 hypercalcaemic patients had significant elevation in plasma PTHrP concentrations [96]. Similar observations were made in stomach cancer where PTHrP is expressed in 77% of gastric adenocarcinomas without humoral hypercalcaemia. In contrast, only 5% of adenomas and none of the nonneoplastic epithelium showed PTHrP expression. Similarly, PTHrP expression was more common in moderately differentiated adenocarcinomas (95.5%) and poorly differentiated adenocarcinomas (100%) than in well-differentiated adenocarcinomas (43%). Furthermore, PTHrP expression was more intense in the deeply invasive portions than in the mucosal carcinomas [97].

It is well established that PTHrP is the major causative agent in MAH associated with a broad range of tumors. However, this is only one aspect of the multiple facets of PTHrP in cancer biology. Indeed, the complex growth factor-like properties of PTHrP have shed new light onto potential roles for this peptide in the regulation of tumor growth and invasion. In support of this, PTHrP expression has been shown to be under the control of numerous growth and angiogenic factors such as transforming growth factor (TGF-β,) epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) [98, 99]. Conversely, PTHrP stimulates the expression of many of these factors in various cell types and behaves as an angiogenic factor in endothelial cells [98, 100, 101]. More recently, PTHrP was shown to promote cell invasion in vitro [102, 103]. These observations clearly suggest a multifunctional role for PTHrP in cancer biology. Its functions include (1) regulation of tumor cells growth, differentiation, and progression; (2) induction of progression of osteolytic bone metastasis, (3) moderation of tumor cell survival factors and interference with apoptosis signaling pathway.

12.3.3 PTHrP in Breast Cancer Development

Breast cancer is frequently accompanied by PTHrP-induced hypercalcemia in advanced stages of the disease [25], and 50–60% of primary human breast cancer tumors overexpress PTHrP [85, 104, 105]. Several retrospective studies suggested that breast cancer patients with PTHrP-positive primary tumors were more likely to develop bone metastases compared to breast cancer patients with PTHrP-negative tumors

[85, 104–106]. PTHrP is expressed in 68% of surgically excised early breast cancers, compared with 100% of bone metastases [107], and 50% of cases of early breast cancer expressed the PTHrP receptor in contrast to 81% of cases of bone metastases. PTHrP expression without concomitant expression of its PTH1R receptor in primary tumors correlated with a reduced disease-free survival with a mortality rate of 6%, while co-expression of both predicted the worst clinical outcome at 5 years with a mortality rate of 32% [107]. A similar observation was noted regarding the differential expression of PTHrP isoforms in different stages of breast cancer. The levels of the 1–139 isoform mRNA was much higher in the tumors of patients who later developed metastases than in those of patients who developed no metastases. This mRNA isoform was also more abundant in breast tumors from patients who developed bone metastases than in those of patients who developed metastases in soft tissues. In contrast, the amounts of the 1–141 isoform mRNA in these groups of tumors were similar [87].

A prospective study conducted by Dr Kremer's team in patients with malignancy-associated hypercalcemia (including breast cancer patients) indicated that elevated circulating levels of PTHrP is an indicator of poor prognosis and is associated with reduced survival (Fig 12.3) [108]. Intriguingly, however, a retrospective clinical study of breast tumors collected at surgery suggests a better outcome and survival in patients whose primary tumor overexpresses PTHrP [109, 110].

To shed light on this controversy, Sato and co-workers treated animals presenting symptoms similar to those of HHM patients with a humanized anti-PTHrP antibody. The animals showed significant improvement in hypercalcemia and cachexia after antibody treatment [111]. Furthermore, administration of this antibody in nude mice injected intra-cardiac with the human breast cancer cell line MDA-MB-231 reduced the ability of these cells to form bone metastases [112]. Animal studies using mice transplanted with human tumors expressing PTHrP suggest that the humanized anti-PTHrP antibody could be an effective and beneficial agent for patients with HHM. In vitro, overexpression of PTHrP in the human breast cancer cell line MCF-7 caused an increase in mitogenesis, whereas the inhibition of endogenous PTHrP production resulted in decreased cell proliferation [113]. Enhanced bone tumor growth was also observed when the MCF-7 cells were transfected to overexpress PTHrP. Taken together, these results point to the pro-tumorigenic effects of PTHrP [94].

12.3.4 Role of PTHrP in Breast Cancer Metastasis to Bone

The bone matrix contains large amounts of growth factors such as type I collagen, insulin-like growth factors (IGFs), transforming growth factor α and β (TGF α and β), fibroblast growth factors (FGF-1 and -2), platelet derived growth factors (PDGFs) and bone morphogenic proteins (BMPs) [114].

The bone remodeling cycle involves a series of highly regulated steps which depend on the interactions of two cell types, the mesenchymal osteoblastic lineage and the hematopoietic osteoclastic lineage. Osteoblasts synthesize some of the growth factors stored in the matrix during bone formation. Most growth factors are released in active form into the marrow when bone is degraded during osteoclastic bone resorption. The resorption areas provide a fertile microenvironment for tumor cell colonization and proliferation [115, 116].

The inner structure of bone consists of bone marrow which is multicellular and contains hematopoietic stem cells and stromal cells. Hematopoietic stem cells differentiate into any type of blood and immune cells including macrophages, lymphocytes and osteoclasts. Stromal cells support the differentiation of the hematopoietic stem cells but importantly, have the ability to differentiate into osteoblasts [117]. In addition, physical factors within the bone microenvironment, including low oxygen levels, acidic pH and high extracellular calcium concentrations may also enhance tumor growth [118]. Furthermore,

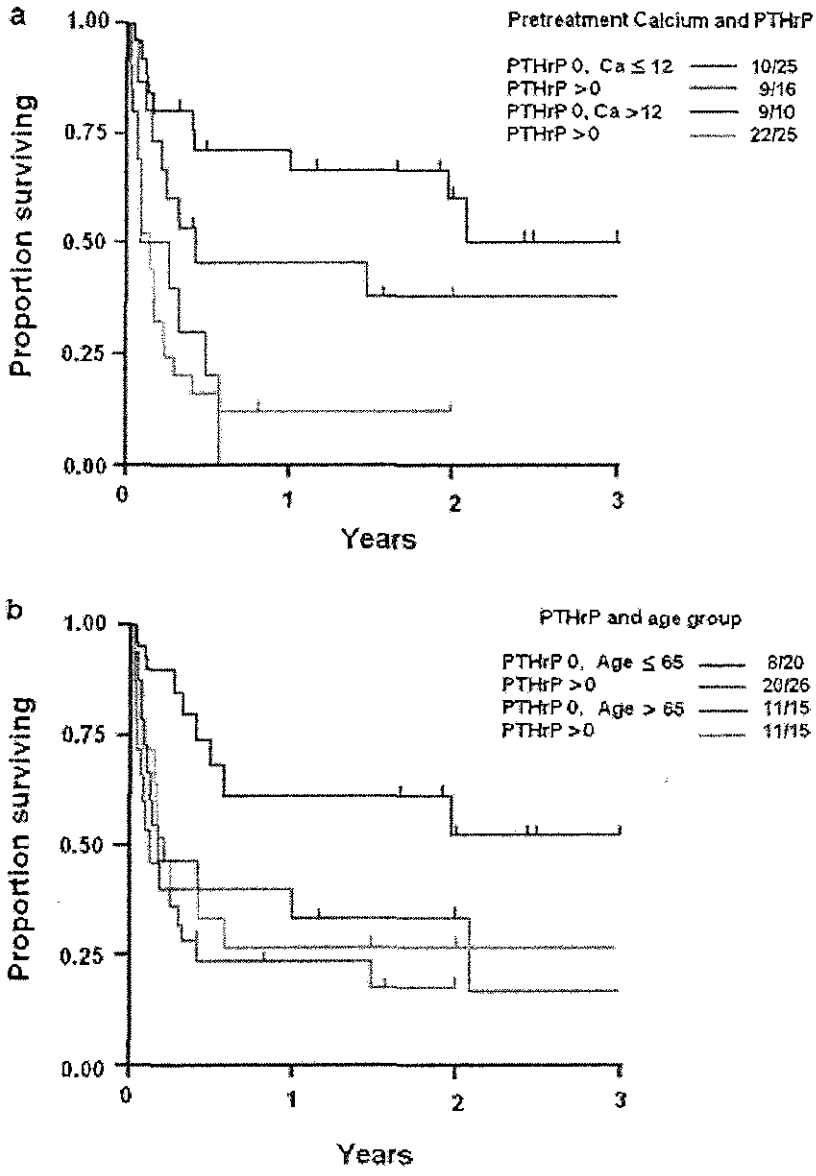

Fig. 12.3 Serum levels of PTHrP and survival of patients with MAH. (a) Survival in 76 hypercalcemic cancer patients, by PTHrP status and pretreatment calcium levels. Numbers shown in the inset are total numbers of deaths/number of patients at baseline. Numbers of patients at risk were 40 at 100 days, 22 at year 1, and 3 at year 3. (b) Survival in hypercalcemic cancer patients by PTHrP status and age group. The numbers of patients at risk were 41 at 100 days, 22 at year 1, and 3 at year 3. CA ≤ 12 = pretreatment serum calcium levels 10.3 to 12 mg/dl; CA > 12 = pretreatment calcium levels > 12 mg/dl; PTHrP 0 = PTHrP not elevated; PTHrP > 0 = PTHrP elevated. PTHrP and calcium levels are two independent prognostic factors for patient survival, and the effect of PTHrP is only significant in patients younger than 65

when the tumor cells stimulate osteoclastic bone resorption, the bone microenvironment becomes more enriched in bone-derived growth factors that enhance survival of the cancer and disrupt normal bone remodeling, resulting in bone destruction.

Metastasis is the spread and growth of tumor cells to distant organs, and represents the most devastating attribute of cancer. The common sites for metastatic spread of breast cancer are bone, lung and liver [119]. However, certain cancers will form metastases in specific organs or tissues at higher frequencies than predicted by blood flow patterns alone. For instance, breast, prostate, lung cancer and multiple myeloma frequently metastasize to bone. Bone metastases are often

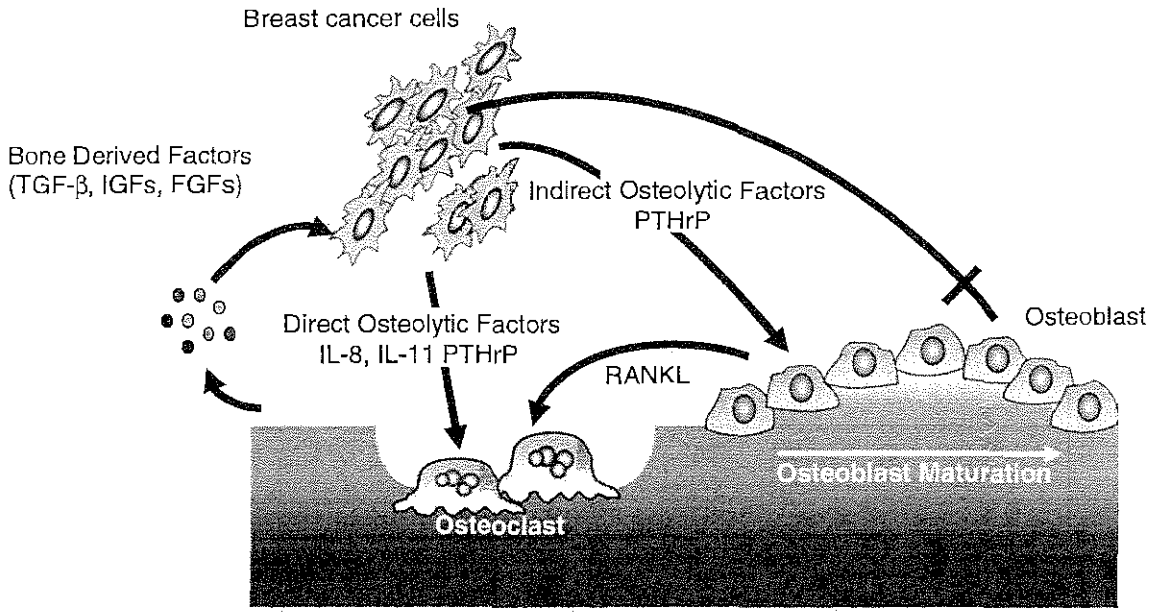

Fig 12.4 Interactions driving osteolytic metastases

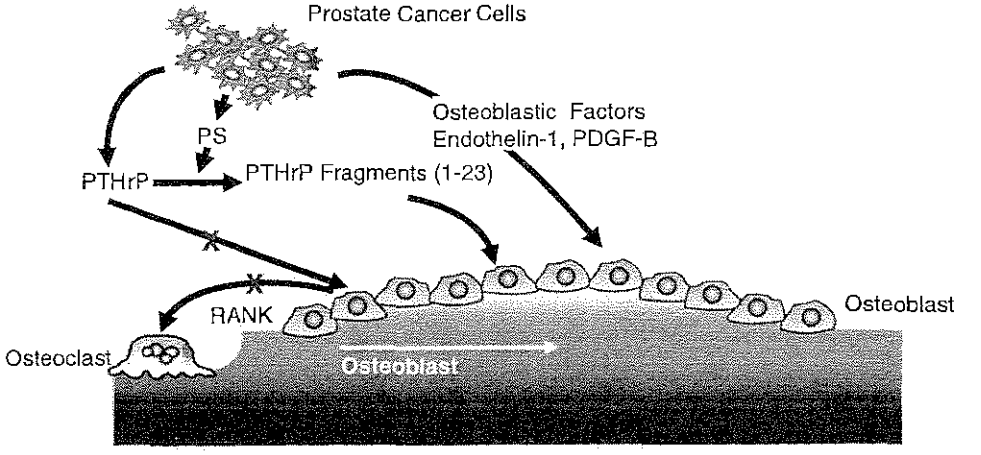

Fig 12.5 Interactions driving osteoblastic metastases

broadly classified as osteolytic (proceeding through bone destruction) (Fig. 12.4) or osteoblastic (proceeding through aberrant bone formation) (Fig. 12.5). Osteolytic bone lesions are typical of breast and lung carcinoma as well as hematological malignancies such as multiple myeloma. On the other hand, tumors in bone may stimulate new bone formation resulting in osteoblastic bone metastasis, a situation most often associated with prostate cancer although also encountered in breast cancer. The osteolytic-osteoblastic distinction is not absolute, as many

patients with bone metastases have both types of lesions and individual metastatic lesions can contain both osteolytic and osteoblastic components. A dysregulation of the normal bone remodeling process is encountered in both types of lesions [120].

According to the Stanley Paget "seed and soil" hypothesis, tumor cells (seed) invading bone provide growth factors that activate the bone microenvironment (soil), which in turn produces growth factors that feed the tumor cells, creating a vicious cycle of destructive mutual cooperation [121].

PTHrP has been shown to play a key role in the osteoclastic bone resorption resulting from breast cancer metastasis to bone because it activates local bone turnover and consequently participates actively in the vicious cycle described above [117, 122, 123]. It has to be noted that PTHrP expression by breast cancer at metastatic sites differs dramatically from expression at primary sites. In fact, only 50% of primary breast cancers express PTHrP, whereas 92% of metastases of breast cancer to bone produce the peptide [124]. The increased local PTHrP stimulates RANKL expression and inhibits OPG secretion by osteoblasts. RANKL binds to its receptor RANK (expressed on osteoclasts) and enhances the differentiation and fusion of active osteoclasts in the presence of the macrophage-colony-stimulating factor. Bone-derived TGF-β, IGFs and FGFs released as a consequence of osteoclastic bone resorption stimulate tumor production of PTHrP via different receptors present in the cancer cells (PKC, MEK, P38 MAPK and Akt signaling pathway) [121, 125, 126]. Tumor cells might contribute to the vicious cycle by producing growth factors and cytokines which in turn sustain tumor growth [127]. It is, however, unclear whether the predilection of these cancers for spreading to bone results from the induction of PTHrP in the bone microenvironment, or whether tumors that produce PTHrP are more likely to metastasize to bone [124]. In contrast to its well-characterized role in bone metastasis development, the role of PTHrP in tumor progression outside the skeleton remains controversial.

12.4 *Pthrp* Gene Ablation in Mammary Epithelial Cells and Its Consequences on Tumor Initiation, Growth and Metastasis

12.4.1 Rationale

PTHrP is expressed in normal epithelial cells but its expression increases in breast cancer and becomes associated with multiple metastatic lesions and reduced survival. It is, however, still unknown whether PTHrP overexpression is simply a consequence of tumor progression, or whether it is mechanistically linked to the tumor progression process from initiation to metastasis. In order to shed light on this relationship, we ablated the *Pthrp* gene in mammary epithelial cells and determined the consequences of this ablation on tumor initiation, growth and metastasis.

We used a model in which PTHrP is specifically excised in mammary epithelial cells using the cre-loxPsystem. In this model, it is important to note that Cre is expressed shortly after birth (around 10 days) [128] but not during embryonic development [129], making the system suitable for the study of PTHrP roles in mammary development during puberty as well as in tumor initiation arising from the normal mammary epithelium.

12.4.2 MMTV-PyMT Breast Cancer Mouse Model

In the MMTV-PyMT transgenic mouse model [130], expression of the oncoprotein polyoma middle T antigen (PyMT) is under the control of the mouse mammary tumor virus long terminal repeat (LTR) and its expression is restricted to the mammary epithelium and absent from myoepithelial and surrounding stromal cells.

PyMT, a membrane-attached protein, is encoded by the small DNA polyoma virus. PyMT is not normally expressed in human breast tumor cells, but when overexpressed in the mammary epithelium of transgenic mice, it acts as a potent oncogene because its product (MT oncoprotein)

binds various receptors and activates several signal transduction pathways, including those of the Src family kinase, ras and the PI3K pathways, which are frequently activated in human breast cancers [131]. Src phosphorylates Y315, which in turn directly activates the p85/p110/PI3K complex, which then activates PDK1 and 2 and results in AKT phosphorylation and activation [131]. Previous studies have shown that PTHrP can activate both ras [132] and AKT [133, 134] signaling pathways.

12.4.3 Tumor Progression from Hyperplasia to Metastastic Carcinoma

Mammary hyperplasia can be detected in this animal model as early as 4 weeks of age. The hyperplasia then progresses to adenoma in 6 weeks, to early carcinoma in 9 weeks and to late carcinoma 12 weeks, with pulmonary metastasis present in 100% of animals. The MMTV-PyMT mouse model of breast cancer is characterized by a high incidence of lung metastasis with highly reproducible progression kinetics. Increased metastatic potential has been shown to depend on the presence of macrophages in primary tumors and on the establishment of a chemoattractant paracrine loop of colony-stimulating factor-1 (CSF-1) and EGF ligands between macrophages and tumor cells [135]. Although PyMT transgenic mice do not develop bone metastasis per se, metastatic cells are found in the bone marrow relatively early and continue to grow in later stages without evidence of bone metastasis [136]

12.4.4 Conditional Knockout of *Pthrp* in Mammalian Epithelial Cells of the MMTV-PyVMT Transgenic Mouse Model

Disruption of the *Pthrp* gene in the mammary epithelium of the PyVMT transgenic mouse model produces mice that are homozygous (*PyVMT-Pthrp*$^{flox/flox}$) or heterozygous (*PyVMT-Pthrp*$^{flox/+}$) for the floxed *Pthrp* allele. Both groups of animals possess two active *Pthrp* alleles, whether flanked by flox sequences or not. These mice were crossed with a separate strain expressing Cre recombinase under the control of the MMTV promoter that targets Cre expression to the mammary epithelium. Excision of flox-bordered essential *Pthrp* sequences renders the gene nonfunctional. The resulting homozygous mice (*PyVMT-Pthrp*$^{flox/flox}$; *Cre*$^{+}$) therefore express no PTHrP in the mammary epithelium, while the heterozygous mice (*PyVMTPthrp*$^{flox/+}$;*Cre*$^{+}$) present lowered levels of PTHrP expression because they retain one active *Pthrp* allele.

A significant consequence of reduction or elimination of PTHrP expression in the mammary epithelium of the offspring is a marked delay in tumor onset. 100% of control animals (normal PTHrP levels) present tumors by day 55, while heterozygous (PTHrP haploinsufficiency) and homozygous animals (absent PTHrP) reach this percentage by days 77 and 85, respectively.

Metastatic spread to lungs was similarly reduced independent of tumor size, illustrating the crucial importance of ablating PTHrP signaling to prevent breast cancer progression and metastasis [137].

Other Cre/lox studies indicate ablation of floxed sequences in only 90% of the cells, leaving 10% of the cells to potentially express the knock-out protein. In order to overcome the problem of residual PTHrP expression, we isolated cells from *PyVMT Pthrp*$^{flox/flox}$;*Cre*$^{-}$ tumors, transfected them with an adenoCre-GFP (or control adeno-GFP) construct, subcultured the cells and purified them by flow cytometry to obtain pure populations of Cre^{+} (or control Cre^{-}) cells with complete or no *Pthrp* ablation. When these cells were transplanted into the mammary fat pad of healthy syngeneic mice, tumor onset was significantly delayed post-tumor implantation in adeno-Cre animals compared to adeno-GFP controls [137]. Tumor growth was also significantly delayed in the adenoCre mice. Metastatic tumor cells were detectable in the bone marrow of adenoCre animals during killing, confirming that this model can be used to examine natural progression of breast cancer from the primary site to the skeleton. The ablation of *Pthrp* was also observed

to inhibit G0/G1 to S transition in tumor cells, to enhance tumor cell apoptosis (increased TUNEL staining and decreased Bcl-2 expression) and to decrease Akt1 and increase Akt2 expression [137]. From these observations, we concluded that PTHrP is critical for the initiation of mammary tumorigenesis in vivo, and that ablation of PTHrP expression in mammary epithelial tumor cells inhibits tumor growth and metastasis by several mechanisms.

12.5 Summary and Conclusion

PTHrP has been associated with cancer as the causative agent for malignancy-associated hypercalcemia, and with progression of metastases in the skeletal environment. We show that PTHrP is involved in breast cancer initiation, growth and metastasis, and we suggest that PTHrP plays a role in a number of critical checkpoints for PyVMT, which points to a novel role as a facilitator of oncogenes and emphasizes the importance of attempting its targeting for therapeutic purposes.

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